UNIVERSITÉ CATHOLIQUE DE LOUVAIN

SECTEUR DES SCIENCES DE LA SANTÉ
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TRANSDIFFERENTIATION OF PANCREATIC DUCT CELLS TO β-CELLS IN ABSENCE OF THE TRANSCRIPTION FACTOR HNF6

ERIC A. HEINEN

Supervisor : Prof. Patrick Jacquemin
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Thesis submitted in fulfillment of the requirements for the degree of PhD in Biomedical and Pharmaceutical Sciences (Docteur en Sciences Biomédicales et Pharmaceutiques)

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Summary

A promising way to cure diabetes is to implant in vitro produced β-cells, or to induce β-cell regeneration in vivo. Multiple attempts have been made to coax different cell types into β-cells. One of these cell types is the pancreatic duct cell, but its potential as a β-cell precursor remains unclear.

In this work we examine the plasticity of duct cells in mice deficient for Hepatocyte Nuclear Factor 6 (HNF6), a transcriptional regulator of pancreatic cell differentiation. In *hnf6*-/- mice, the number of β-cells is reduced at birth, yet it subsequently undergoes a partial recovery. We show in these mice that a postnatal β-cell neogenesis occurs. Neogenesis neither results from a higher proliferation rate of mature β-cells nor from delayed differentiation of β-cell precursors. The presence of transition cells that co-express insulin and duct markers, as well as genetic lineage tracing, indicate that duct cells transdifferentiate postnatally to β-cells in *hnf6-/-* mice.

Therefore, our results provide evidence that in the absence of the transcription factor HNF6, duct cells have the potential to generate β-cells. This suggests new strategies for the production of β-cells from duct cells.

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1. Introduction

1.1. Functions of the pancreas

The pancreas combines exocrine and endocrine functions. Its anatomy and structure are shown in Figure 1 and Figure 2.

The exocrine pancreas produces and secretes bicarbonate and a variety of digestive enzymes which include proteases, amylases, lipases and nucleases (Slack, 1995). Most are secreted as inactive precursors and become activated in the duodenum.

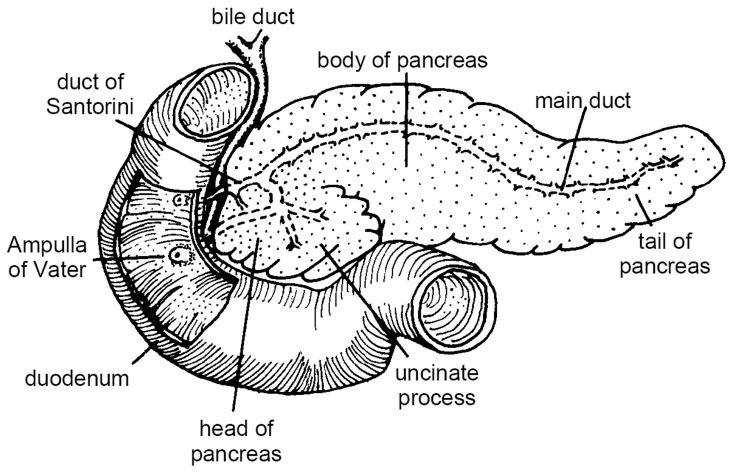

Figure 1. Anatomy of an adult human pancreas. *Adapted from Slack (1995).*

The enzymes are synthesized in acinar cells, stored in secretory (zymogen) granules, and released into the acinar lumen upon stimulation by secretin,

cholecystokinin and gastrin, and also by neural stimuli. The secretions are collected by the intralobular ducts, whose epithelium secretes bicarbonate, to form the final pancreatic juice. The latter is drained by larger interlobular ducts coalescing into the main pancreatic duct (duct of Wirsung), which is connected to the duodenum via the ampulla of Vater. In the human, the terms head, neck, body and tail are used to designate regions of the organ from proximal to distal, while in rodents the shape of the pancreas is rather less well defined.

The endocrine cells are grouped into the islets of Langerhans. They are involved in the regulation of glycemia (secretion of glucagon, insulin) and pancreatic exocrine and endocrine secretion (secretion of somatostatin, pancreatic polypeptide, ghrelin). The hormones are produced by five different cell types: α-cells (glucagon), β-cells (insulin), δ-cells (somatostatin), PP-cells (pancreatic peptide), and the recently described ε-cells (ghrelin) (Prado et al., 2004; Wierup et al., 2002). The majority of endocrine cells are β-cells which represent 54% of the human islets cells (75% in mice), α-cells make up for 35% (19% in mice) and δ-cells, 11% (6% in mice) (Brissova et al., 2005; Cabrera et al., 2006). The contribution of PP-cells and ε-cells is less than 2%. These numbers may vary between individuals, especially in humans. In rodents, the different endocrine cell types are segregated within the islets, such that the β-cells lie in its centre and the other types at its periphery. In humans, β-cells are not clustered, and most (71%) show associations with other endocrine cells, suggesting unique paracrine interactions in human islets (Cabrera et al., 2006). Furthermore, in contrast to mice, all endocrine cell types are closely associated with the islet's microcirculation and there is no particular order in the alignment of endocrine cells along the blood vessels. As a consequence of these morphological differences, human and mice islets have a slightly distinct physiology.

The pancreas has a rich blood supply, the arterial blood passing in each lobule first to the islets and then to the adjacent acini (Slack, 1995). There is also an extensive lymphatic drainage, and a rich sympathetic and parasympathetic nerve supply. Smooth muscle is found around the larger ducts and in the sphincter muscles of the ampulla. The fibroblastic, lymphatic and smooth muscle components of the pancreas are presumed to arise from the abundant mesenchyme surrounding the embryonic buds.

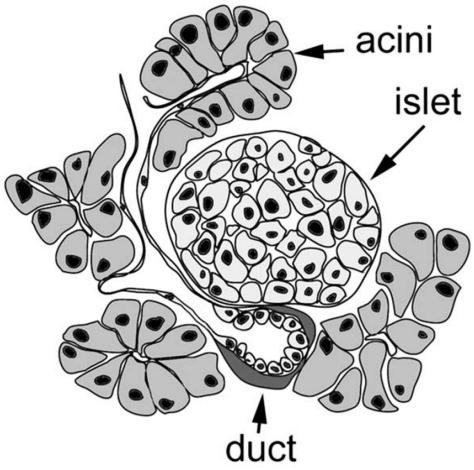

Figure 2. Schematic representation of the structure of an adult pancreas. *From Gu et al. (2003).*

1.2. Morphogenesis of the pancreas

In mammals, the pancreas derives from two cell populations located dorsally and ventrally in the gut endoderm. During embryogenesis, both populations are exposed to different mesodermal-derived structures that are essential for their development. For the dorsal pancreas, these are respectively the notochord, the aorta and the pancreatic mesenchyme (Kim and MacDonald, 2002) (Figure 3). For the ventral endoderm, these are the septum transversum, the cardiogenic mesoderm, and the pancreatic mesenchyme. At approximately 9.5 days of gestation in mice (E9.5, where E0.5 is defined as noon of the day when a vaginal plug is detected), the pancreatic endoderm evaginates into the overlying mesenchyme and gives rise to the dorsal pancreatic bud at the 25-somite stage (E10) (Pictet and Rutter, 1972; Wessells and Cohen, 1967). Ventrally, a bud appears slightly later, at about the 30-somite stage (E10.25) (Pictet and Rutter, 1972). It develops morphologically like the dorsal bud (Spooner et al., 1970). In the buds, cells proliferate to give rise to a population of progenitors for acini, islets and ducts (Jorgensen et al., 2007; Kim and MacDonald, 2002).

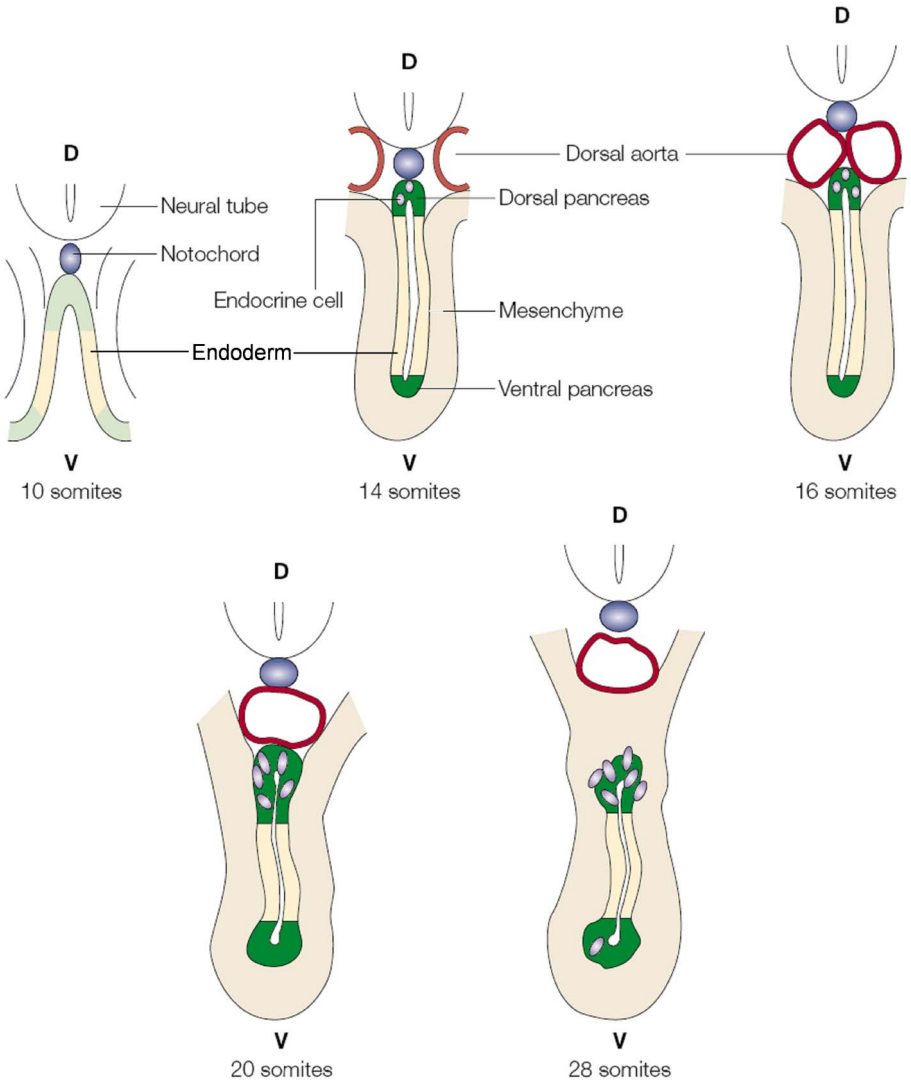

Figure 3. Early stages of pancreatic organogenesis. *The 10-somite stage corresponds to embryonic day 8 (E8), whereas the 28-somite stage roughly corresponds to E10. D = dorsal; V = ventral. Adapted from Edlund (2002).*

As a result of both gut rotation and elongation of the dorsal and ventral stalks, both parts of the pancreas come into contact and merge at E16-E17 to become indistinguishable (Pictet and Rutter, 1972) (Figure 4). This also leads to fusion of the ventral duct with the distal portion of the dorsal duct, to constitute the duct of Wirsung. The proximal portion of the dorsal duct persists as the smaller accessory duct of Santorini.

It has been demonstrated by genetic lineage-tracing experiments that a specific domain, located at the tip of the branching pancreatic tree, contains multipotent progenitors that produce exocrine, endocrine, and also duct cells (Zhou et al., 2007).

The outgrowth of multipotent tip cells leaves behind differentiated progeny that form the trunk of the branches.

The first endocrine cells, detectable by immunolabeling, are glucagon-positive and PP-positive cells (Herrera et al., 1991). They appear at E10.5 in the dorsal bud and at E11.5 in the ventral bud. Insulin-expressing cells become detectable at E11.5 in the dorsal bud and two days later in the ventral bud. Somatostatin-positive cells can be found from E13.5 on in both dorsal and ventral pancreas.

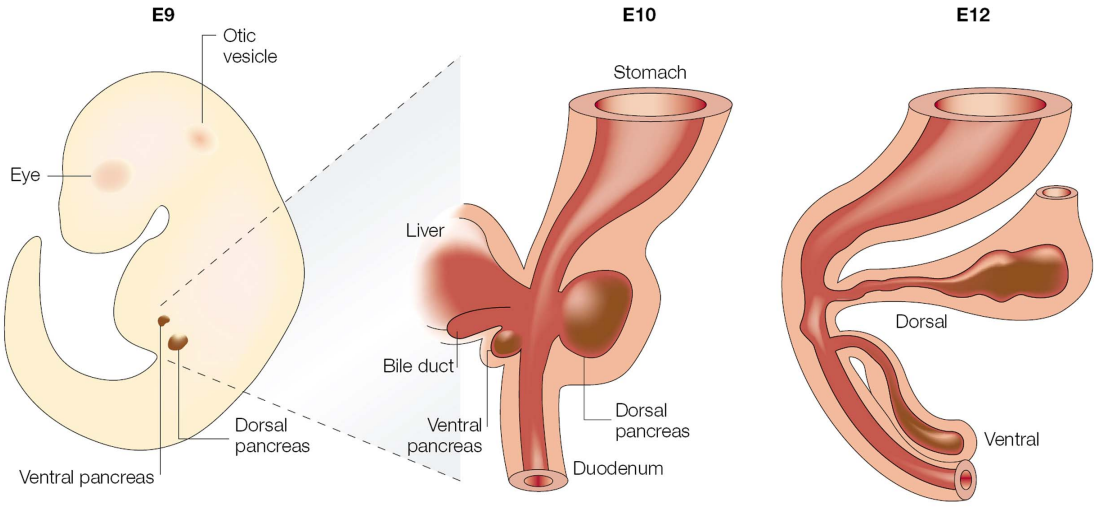

Figure 4. Schematic representation of the mouse pancreas at E9, E10 and E12. *Adapted from Edlund (2002).*

From E13.5 until E18.5, the pancreas undergoes a wave of massive endocrine and exocrine cell differentiation. During this phase, called "secondary transition" the number of endocrine cells increases rapidly and β-cells become the most abundant fraction of them (Pictet and Rutter, 1972). Morphologically, the early endocrine cells stay integrated in the pancreatic epithelium, which consist of a highly folded epithelial sheet. These epithelial cells maintain critical tight junction connections and retain E-cadherin positivity (Gittes, 2009). As development goes on, the endocrine cells undergo a key morphologic transition: they leave the epithelium and loose tight junctions. This epithelial to mesenchymal transition has been postulated to entail a change in cell division polarity, changing from perpendicular to the basement membrane to parallel to the basement membrane (Pictet and Rutter, 1972). From E14 to E18, the new endocrine cells accumulate along the ducts and blood vessels in a

cord-like pattern. In the perinatal phase, they coalesce into aggregates that represent the first islets of Langerhans, which can be detected from E18.5 on (Herrera et al., 1991).

The vascular system in the pancreatic buds provide oxygen and nutrients to the developing endocrine precursors and ultimately allow endocrine function (Cleaver and Melton, 2003). Neural crest cells migrate into the developing pancreas and, as they develop into neurons, affect the numbers of β-cells (Nekrep et al., 2008). Hence, the co-differentiation of the stromal environment with that of the pancreatic progenitors is crucial because of stimulatory roles of endothelial cells and neural crests.

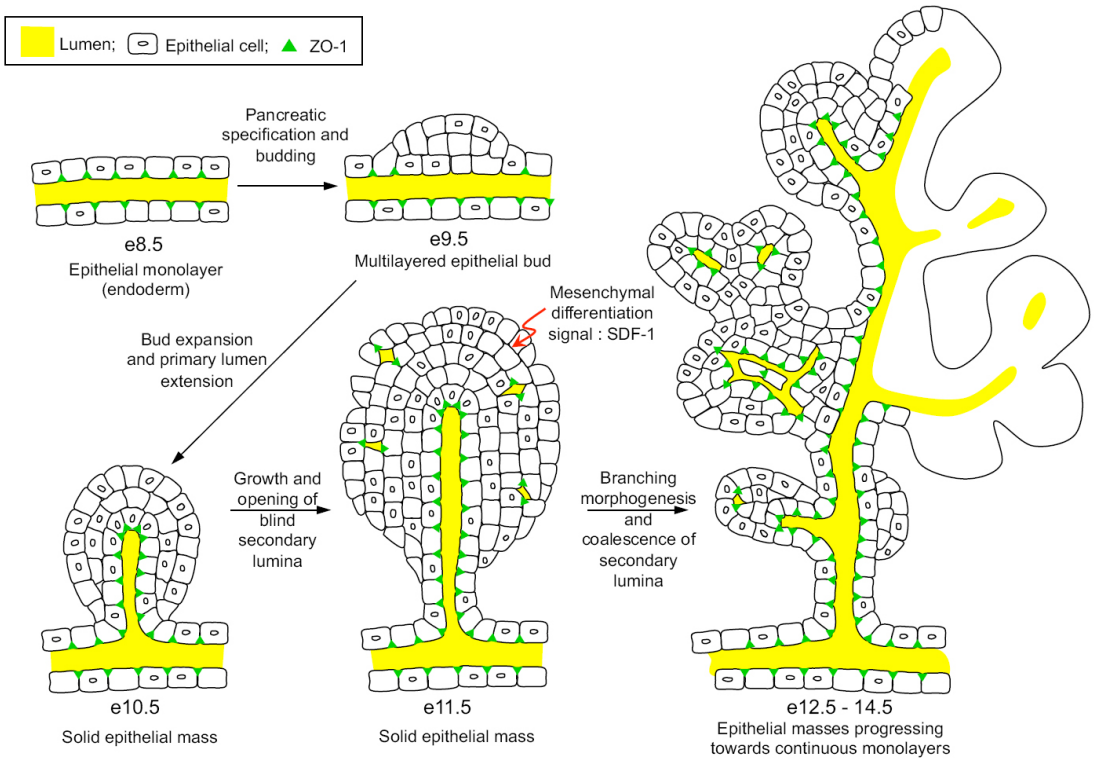

Figure 5. Development of the pancreatic duct network. *Until E10.5 the pancreatic buds consist of a compact multilayered mass of epithelial cells. At E11.5, the first microlumens are scattered throughout the bud. They expand and coalesce to form an interconnected tubular network. The mature ducts are composed of a monolayered epithelium where all the cells are polarized (characterized by apical tight junctions containing ZO-1). From Hick et al. (2009).*

In the acinar cells, the secondary transition is characterized by an exponential increase in enzyme gene expression, development of large amounts of rough endoplasmic reticulum, and formation of zymogen granules (Pictet and Rutter, 1972). Due to the presence of the latter, the pancreas becomes opaque. At about E14.5, acini and ducts become clearly visible as histologically differentiated structures, and amylase becomes detectable by immunostaining (Slack, 1995).

The development of pancreatic ducts begins at E11.5 (Hick et al., 2009; Kesavan et al., 2009). Before this stage, the pancreatic buds are multilayered and have a globular shape (Figure 5). Most epithelial cells are not polarized and lack apico-basal cell polarity. At E11.5-E12.5, small dots of mucin-1, an apical marker, appear scattered throughout the epithelium, indicating the formation of early microlumens. These microlumens subsequently expand and coalesce to form an interconnected tubular network. At E15.5, the ducts are mature and delineated by a monolayered epithelium surrounded by mesenchyme. All epithelial cells have become polarized and show apical, lateral and basal domains. The ductal network further grows by lateral branching (Puri and Hebrok, 2007).

1.3. Molecular regulation of pancreas development

1.3.1. Pancreas specification

The developmental biology of the pancreas has been extensively reviewed by Gittes et al. (2009). The pancreatic specification starts in the dorsal prepancreatic endoderm, which lies in close contact to the notochord. Unlike adjacent endoderm in the gastric and duodenal anlagen, this region does not express Sonic hedgehog (SHH), a member of the hedgehog ligand family (Apelqvist et al., 1997). Signals from the notochord, that include activin and fibroblast growth factor (FGF), are thought to

repress SHH in the endoderm and initiate pancreas development (Apelqvist et al., 1997; Hebrok et al., 1998; Hebrok et al., 2000; Kim et al., 1997). It has also been shown that retinoic acid, secreted by the surrounding mesenchyme, is required for dorsal pancreas specification (Chen et al., 2004; Martin et al., 2005; Molotkov et al., 2005; Stafford et al., 2004; Stafford and Prince, 2002).

The ventral pancreas has no contact with the notochord. This region is devoid of SHH and thus is biased toward pancreas. Morphogenesis of the ventral pancreatic bud and the adjacent liver bud is shaped by signals from the cardiac mesoderm and the septum transversum. They produce respectively FGFs and bone morphogenic proteins (BMPs) which both act as pro-hepatic signals. Ventral pancreas development is initiated during foregut closure, when lateral ventral endoderm cells move caudally to the cardiac domain and hence escape the FGF signaling. Consequently, ventral pancreas formation has been considered as a developmental "default" pathway (Bort et al., 2004; Calmont et al., 2006; Deutsch et al., 2001; Jung et al., 1999; Rossi et al., 2001; Shin et al., 2007; Wandzioch and Zaret, 2009).

The newly specified dorsal and ventral pancreatic endoderm is initially marked by the expression of the homeodomain transcription factor PDX1 (IPF1) which is crucial for pancreatic development (Gu et al., 2002). In mice deficient in PDX1, pancreatic development is severely affected (Jonsson et al., 1994). In absence of PDX1, no ventral bud develops, and the dorsal bud remains severely hypoplastic (Ahlgren et al., 1996). From E8.5-E9.0, PDX1 marks the pancreatic endoderm before it visibly thickens (Ahlgren et al., 1996; Guz et al., 1995; Offield et al., 1996). This corresponds to the classically defined period of pancreatic specification (Wessells and Cohen, 1967). PDX1-positive cells represent progenitors of all the mature pancreatic cell types, including duct, islet and acinar cells (Gu et al., 2002). Over the next several days of development, its expression expands to the posterior stomach, duodenum and bile duct (Ahlgren et al., 1996; Offield et al., 1996). Although PDX1 is recognized as the earliest and most specific gene expressed in the pancreas (Ohlsson et al., 1993), other factors such as HB9 (HLXB9), Hex (HHEX), HNF6 (Onecut1), HNF1β (vHNF1, TCF2), HNF3β (FOXa2), SOX9, and PROX1 are known to precede PDX1 expression in mice (Bort et al., 2004; Burke and Oliver, 2002; Dufort et al., 1998; Grapin-Botton et al., 2001; Harrison et al., 1999; Haumaitre et al., 2005; Jacquemin et al., 2003; Li et al., 1999; Poll et al., 2006; Seymour et al., 2007) . However, these

genes are less specific of the pancreas than PDX1 (Jorgensen et al., 2007). HNF6, HNF1β, HNF3β, and SOX9 act together with PDX1 in an interwoven network during pancreas development. HNF6, which is expressed in the gut endoderm, is regulated by HNF1β, expressed throughout the early endoderm (Barbacci et al., 1999; Haumaitre et al., 2005; Lemaigre et al., 1996; Poll et al., 2006). HNF6 in turn regulates HNF3β, which, along with HNF3α (FOXA1), is a major regulator of PDX1 expression during pancreas specification (Gao et al., 2008; Wu et al., 1997). PDX1 can also be stimulated directly by HNF6 (Jacquemin et al., 2003). Furthermore, in vitro experiments suggest that SOX9 can regulate HNF6, HNF1β and HNF3β expression (Lynn et al., 2007).

During development, the dorsal pancreatic bud becomes adjacent to the endothelial cells of the forming aorta which is the source of secondary inducers, promoting pancreatic development (Lammert et al., 2001, 2003a; Matsumoto et al., 2001; Yoshitomi and Zaret, 2004). The signals from the endothelial cells allow the growth of the pancreatic bud, maintain PDX1 expression and induce the expression of the transcription factor PTF1a (p48). This transcription factor also plays an important role in the early specification of pancreatic progenitor cells. It is first expressed at E9.0, shortly after the onset of PDX1 expression, in cells from the foregut endoderm, that will give rise to dorsal and ventral pancreas (Burlison et al., 2008; Krapp et al., 1998). There is evidence that the co-expression of PDX1 and PTF1a in progenitor cells instructs them toward a pancreatic fate (Afelik et al., 2006; Fukuda et al., 2006; Jarikji et al., 2007; Sumazaki et al., 2004). Similar to PDX1 null mutants, mice deficient in PTF1a only develop an aborted dorsal pancreatic bud and a very small ventral bud (Kawaguchi et al., 2002; Krapp et al., 1998). When PTF1a is knocked down in zebrafish and *Xenopus* embryos, acinar cells are absent (Afelik et al., 2006; Lin et al., 2004). It has been shown that FGF10 signaling from the mesenchyme surrounding the dorsal pancreatic bud is necessary to sustain PTF1a expression (Jacquemin et al., 2006).

In summary, the developmental progression at the stages from E8.5 to E10.5 defines the pancreas as an organ. In this time window, a specific transcription factor combinatorial code, which distinguishes the pancreatic cells from any other cell type in the embryo, becomes consolidated (Jorgensen et al., 2007).

Interestingly, contrary to the liver, it seems that the final size of the pancreas is determined by the original number of progenitor cells already present at this early stage (Stanger et al., 2007). When pancreas development progresses, and more and more cells differentiate towards a specific lineage, multipotent progenitors are located at the tips of the branching ducts. These "tip cells" are characterized by the expression of the transcription factors PDX1, PTF1a and c-MYC, and of the enzyme carboxypeptidase A (CPA) (Zhou et al., 2007). The tip cells divide and give rise, on one hand, to epithelial outgrowths that differentiate to exocrine acini, and, on the other hand, to the trunks of the branched ducts, from which endocrine and duct cells differentiate.

1.3.2. Endocrine development

In the mouse pancreas, the first endocrine cells are detected at E10.5 (Herrera et al., 1991). However, it is at E13.5 that a wave of massive endocrine differentiation sets in. The master gene that orchestrates the onset of the endocrine differentiation program is a basic helix-loop-helix transcription factor, called Neurogenin3 (NGN3) (Gradwohl et al., 2000; Gu et al., 2002; Jensen et al., 2000a; Schwitzgebel et al., 2000). It is a downstream target of HNF6, SOX9, and HNF1β (Haumaitre et al., 2005; Jacquemin et al., 2000; Lee et al., 2001; Lynn et al., 2007; Seymour et al., 2008). Cells expressing transiently high levels of NGN3 during pancreas development give rise to α, β, δ, and PP-cells (Gu et al., 2002). Each of these endocrine precursors is unipotent and gives rise to one single endocrine cell (Desgraz and Herrera, 2009).

Achieving high expression levels of NGN3 appears to be critical for endocrine commitment, as in mice heterozygous or hypomorphic for NGN3, a significant number of the detected NGN3-positive cells adopt ductal or acinar fates (Wang et al., 2010). Mice deficient in NGN3 do not develop any endocrine cells at all (Gradwohl et al., 2000). NGN3-positive cells are observed in the pancreas as early as E9.5, just before the appearance of the first glucagon-expressing cells. Their number increases until E15.5 and decreases from E17.5 on (Gradwohl et al., 2000; Gu et al., 2002; Jensen et al., 2000a; Schwitzgebel et al., 2000). No cells expressing NGN3 at high levels are found in the pancreas after birth. However, recent findings suggest a

persistent low-level expression of NGN3 in adult β-cells, which seems to be essential for correct endocrine functioning (Wang et al., 2009).

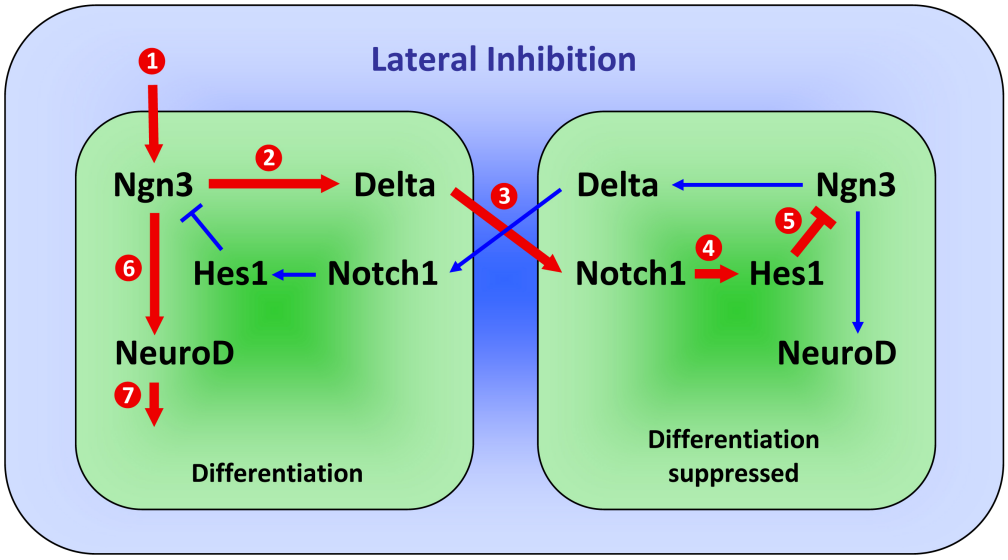

Figure 6. Schematic representation of the Notch signalling pathway involved in the lateral specification of endocrine precursors. *The progressive increase of NGN3 in an epithelial cell (left) activates a Notch ligand (Delta), which binds to the Notch receptor in the neighboring cell (right). This results in the reduction of NGN3 expression via activation of the repressor gene HES1. Adapted from Jensen (2004).*

In an early model of pancreas development, NGN3 would regulate the number of endocrine cells by lateral inhibition within the ductal epithelium via the Notch signaling pathway (Apelqvist et al., 1999; Jensen et al., 2000a; Lee et al., 2001). In this model Notch signaling would occur through a progressive strengthening of the NGN3 expression level in the differentiating cell (Figure 6, left), which induces a weakening of NGN3 expression in neighbors via activation of the repressor HES1 (Figure 6, right) (Artavanis-Tsakonas et al., 1999; Jensen, 2004). The lateral signaling would be mediated through the expression of the Notch ligand Delta, which is controlled by NGN3 (Apelqvist et al., 1999; Jensen et al., 2000a; Lee et al., 2001). However, this model of lateral signaling still awaits experimental validation. Despite this limitation, several experiments addressed subsets of the model. HES1 antagonizes NGN3 expression (Esni et al., 2004), and allows PTF1a to direct the cell via Recombining Binding Protein suppressor of hairless RBP-J toward exocrine

differentiation (Beres et al., 2006; Fujikura et al., 2007; Masui et al., 2007; Obata et al., 2001), and endocrine precursor cells devoid of NGN3 are redirected towards a ductal fate (Wang et al., 2010). Further experiments indicate that sustained Notch signaling activity in the pancreatic epithelium prevents exocrine differentiation (Hald et al., 2003a; Jensen et al., 2000b; Murtaugh et al., 2003a). Moreover, activation of the Notch pathway is necessary in endocrine progenitors to sequester RBP-J so as to avoid differentiation of endocrine progenitors into acinar cells (Cras-Meneur et al., 2009).

Endocrine precursors have a low proliferative activity (Desgraz and Herrera, 2009; Georgia and Bhushan, 2004; Gu et al., 2002; Nekrep et al., 2008) and give rise to post-mitotic cells expressing the islet transcription factors NeuroD and PAX6 (Jensen et al., 2000a). Shortly after NeuroD and PAX6 are expressed, NGN3 is shut off, apparently due in part to auto-repression (Gu et al., 2002; Smith et al., 2004). The paired domain transcription factor PAX6 has been reported to mark all pancreatic endocrine cells from E9.0 on. It continues to be expressed in the mature endocrine cells where it functions as a transcriptional activator of several of hormone genes (Andersen et al., 1999a; Andersen et al., 1999b; Heller et al., 2004; Sander et al., 1997; St-Onge et al., 1997). NeuroD is an immediate downstream target of NGN3 and its expression is dependent of the latter (Gasa et al., 2008; Gradwohl et al., 2000; Gu et al., 2002; Huang et al., 2000; Jensen et al., 2000a). Other examples of downstream targets of NGN3 include IA1, which is activated by NGN3 or NeuroD (Breslin et al., 2002; Mellitzer et al., 2006), IRX1 and IRX2 which are expressed in early pancreatic endoderm and α-cells (Petri et al., 2006), NeuroD2, which is present in embryonic pancreas and α-cell lines (Gasa et al., 2008), and RFX6, which is found in postmitotic endocrine progenitors and maintained in all developing and adult islet cell types (Smith et al., 2010; Soyer et al., 2010).

Many transcription factors are expressed in endocrine cells, showing dynamic expression profiles and playing various functions during endocrine differentiation:

PDX1 : Its expression decreases in the endocrine precursors (Jensen et al., 2000a). However, PDX1 is important for the formation of this cell type, as in PDX1 knock-out mice, NGN3-positive cells were not observed after E9.5 (Burlison et al., 2008). When endocrine cells begin to differentiate toward the β-cell lineage, PDX1

reappears. It is known to be necessary for proper glucose-responsive regulation of insulin synthesis in β-cells (MacFarlane et al., 1994; Marshak et al., 1996). Low levels of PDX1 persist in other endocrine cell types (Guz et al., 1995; Wu et al., 1997).

NKX6.1 : After being expressed in the pancreatic progenitors, NKX6.1 becomes restricted to insulin-positive cells around E15 (Sander et al., 2000), and continues to be expressed in mature β-cells. Mice deficient in NKX6.1 have an 85% reduction in β-cells (Henseleit et al., 2005).

NKX6.2 : Expression first becomes restricted to glucagon-positive and amylase-positive and is then extinguished in the developing pancreas after E15.5 (Sander et al., 2000). Mice deficient in NKX6.2 show normal pancreas development. Double mutants for NKX6.1 and NKX6.2 have a 92% reduction in β-cell and a 65 % reduction in α-cells (Henseleit et al., 2005). This suggests a role for NKX6.2 in α– and β-cell formation, and that NKX6.1 is capable of compensating for the absence of NKX6.2 in these events.

NKX2.2 : It becomes restricted to NGN3-positive cells and persists in most endocrine cells, except the δ-cells (Sussel et al., 1998). Mice deficient in NKX2.2 have no β-cells, 80% reduction in α-cells, a modest reduction in PP-cells and a large increase in ε-cells. This suggests an important role for NKX2.2 in α- and β-cell formation and a repressor function for the development of ε-cells.

HB9 : Its expression decreases until E10.5 but reappears in the developing β cells. At perinatal stages, HB9 becomes restricted to mature β-cells (Harrison et al., 1999; Li et al., 1999; Li and Edlund, 2001). Mice deficient in HB9 have no dorsal pancreas. In the remaining pancreas, β-cells are reduced by 65%. This suggests an important role for HB9 in β-cell and dorsal pancreas formation.

HNF6, SOX9 and HNF1β : They are expressed in NGN3-positive cells, but are not found in the differentiated endocrine cells. They bind to the NGN3 gene and upregulate its expression. Consequently, pancreas deficient in these factors show

absence of, or strong reduction in, endocrine precursors (Gannon et al., 2000; Haumaitre et al., 2005; Jacquemin et al., 2000; Lynn et al., 2007; Seymour et al., 2007; Solar et al., 2009).

HNF3β : Its expression persists to adulthood in the islets. HNF3β appears to have a major function in the maturation of endocrine cells, and is necessary for PDX1 expression in β-cells (Gerrish et al., 2000; Lee et al., 2005; Lee et al., 2002; Sund et al., 2001; Wu et al., 1997).

PROX1 : It remains expressed in NGN3-positive and endocrine cells (Burke and Oliver, 2002; Wang et al., 2005). Mice deficient in PROX1 die at E15 with a small pancreas, that do not generate endocrine cells from the secondary transition (Wang et al., 2005).

The balance between the different endocrine cell types is controlled by the transcription factors PAX4 and ARX. PAX4, an endocrine effector located downstream from NGN3, is a paired-box homeoprotein which by E9.5 is expressed in both ventral and dorsal buds of the developing pancreas (Brink et al., 2001; Sosa-Pineda et al., 1997). The PAX4-positive cell population then expands rapidly, peaking at E13-E15, coinciding with the burst of new insulin-positive cells appearing at the secondary transition (Wang et al., 2004). As the endocrine cells mature, PAX4 expression, which is predominantly found in the developing β-cells, disappear (Smith et al., 1999) (Figure 7). In the absence of PAX4, virtually no β- and δ-cells develop, but the number of α-cells is increased (Sosa-Pineda et al., 1997; Wang et al., 2004). Thus, PAX4 functions early in the development of islet cells to promote the differentiation of β- and δ-cells. PAX4 performs this function by inhibiting the expression of ARX, a homeobox gene acting also downstream of NGN3. At the opposite of PAX4, ARX enhances glucagon-positive cell differentiation (Collombat et al., 2005; Collombat et al., 2003). In the absence of ARX, no α-cells develop and the α-cell precursors seem to be shunted toward the β-cell and δ-cell lineages due to PAX4 expression. Moreover, overexpression of ARX diverts most β-cell and δ-cell precursors toward α-cell and PP lineages, with no changes in the total number of

endocrine cells (Collombat et al., 2007). These results point towards opposing roles for PAX4 and ARX in α/PP cell versus β/δ-cell development.

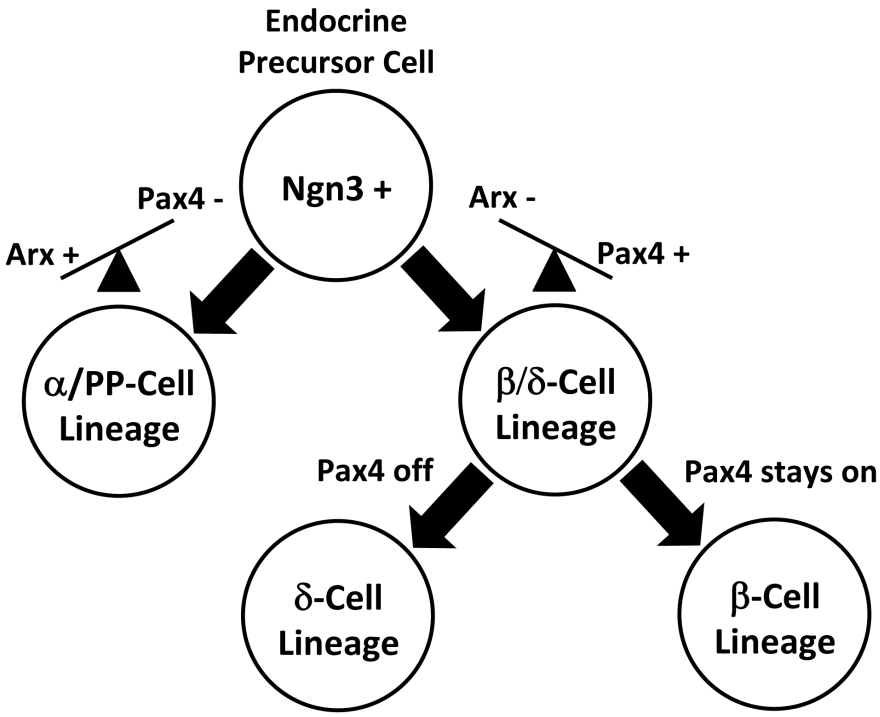

Figure 7. Antagonistic roles of PAX4 and ARX in the fate determination of NGN3-positive cells. *High levels of ARX and the absence of PAX4 favor development of the α/PP-cell lineages, whereas the opposite configuration induces β/δ-cell formation. In the latter scenario, persistent PAX4 expression seems to favor the β-cell lineage, whereas subsequent suppression of PAX4 favors the δ-cell lineage. Adapted from Gittes (2009).*

The basic leucine-zipper transcription factors MAFa and MAFb also play roles in the endocrine differentiation. At early embryonic stages, MAFb is expressed in some NGN3-positive cells, and then in the glucagon and insulin-positive cells (Artner et al., 2006; Gu et al., 2004). Around the secondary transition, it becomes down-regulated in the insulin-positive cells when MAFa appears (Matsuoka et al., 2004; Nishimura et al., 2006). This transition from MAFb to MAFa seems to depend on MAFb function, since MAFb binds and activates the MAFa gene (Artner et al., 2007). MAFb becomes restricted to the α-cells where it controls glucagon gene expression (Artner et al., 2006; Gu et al., 2004; Matsuoka et al., 2004; Nishimura et al., 2006).

MAFa in turn is an activator of insulin gene expression and appears to be specific for developing and mature β-cells (Matsuoka et al., 2003; Olbrot et al., 2002). Its promoter binds to the PDX1, NKX2.2 and HNF3β genes (Raum et al., 2006). Knock-out studies have shown that MAFa is not necessary for β-cell formation, but plays a role in β-cell function in the mature pancreas (Zhang et al., 2005).

Two other factors, ISL1 and MYT1 have also been found to be important for endocrine development. ISL1, a lim homeodomain protein expressed in the early pancreatic epithelium, is cell-autonomously necessary for the development of all endocrine cells (Ahlgren et al., 1997). ISL1-positive cells are postmitotic. It is suggested that ISL1 is located downstream of NeuroD and upstream of PAX6 in the endocrine cascade (Jensen et al., 2000a). In adult pancreas, ISL1 is expressed in all hormone-producing cells of the islets (Ahlgren et al., 1997; Thor et al., 1991).

The transcription factor MYT1 seems to be an important regulator of pancreatic endocrine cell differentiation (Gu et al., 2004). The absence of MYT1 leads to the development of abnormal endocrine cells, expressing multiple hormones (Wang et al., 2007). Its expression pattern in the developing pancreas is similar to the one of NGN3 (Gu et al., 2004; Wang et al., 2007). In fact MYT1 and NGN3 form a feed-forward expression loop and positively regulate each other's expression to promote endocrine differentiation (Wang et al., 2008). MYT1 induces glucagon expression by potentiating NGN3 transcription in pancreatic progenitors. Vice versa, NGN3 protein production induces the expression of MYT1.

Figure 8 shows a summary of the expression pattern of selected transcription factors during β-cell development.

As development progresses and the endocrine cells cluster together to form compact islets of Langerhans, vascularization becomes an issue. It has been suggested that this takes place in two sequential steps (Lammert et al., 2003b): In the first step, signals from blood vessel endothelium induce islet formation next to the vessels, and in the second step, the islets signal to the endothelium. The second step involves paracrine vascular endothelial growth factor A (VEGF-A) signaling to elaborate the interaction of islets with the circulatory system and endothelial cells producing laminins which stimulate insulin gene expression and beta cell proliferation (Lammert et al., 2003a; Nikolova et al., 2006). Recently, it has been demonstrated that oxygen

supply also plays an important role in β-cell differentiation (Heinis et al., 2010). The latter is controlled by oxygen tension through the hypoxia-inducible factor 1α (HIF1α).

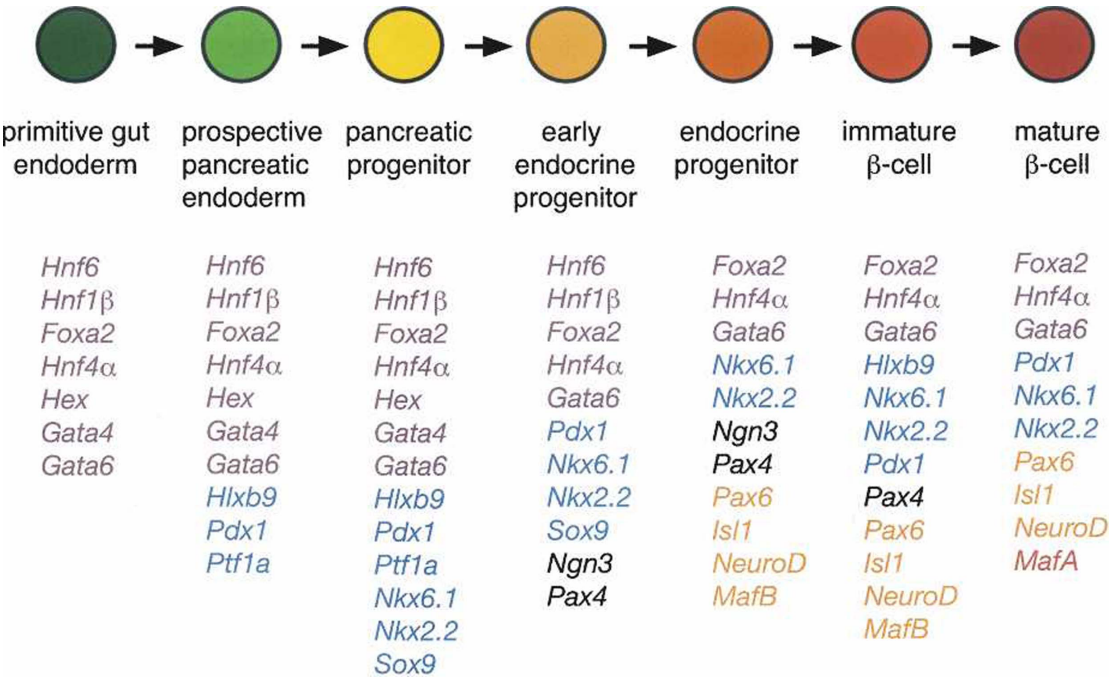

Figure 8. Expression profile of several transcription factors involved in β-cell development from primitive gut endoderm to the mature β-cell. *Factors initially expressed at the same developmental stage are color-coded as follows: gut endoderm = purple, pancreatic endoderm progenitor = blue, early endocrine progenitor = black, endocrine progenitor = orange, β-cell = red. From Oliver-Krasinski and Stoffers (2008).*

1.3.3. Acinar development

Signals from the mesenchyme surrounding the pancreatic bud are critical for the development of acinar tissue. They promote acinar differentiation at the expense of endocrine development (Crisera et al., 2000; Duvillie et al., 2006; Gittes et al., 1996; Li et al., 2004; Miralles et al., 1998). In the absence of mesenchyme, the pancreatic epithelium differentiates toward islets. It appears that contact of the mesenchyme with

epithelium may both enhance HES1 expression and inhibit NGN3 expression via the Notch signaling pathway, favoring non-endocrine lineages over endocrine lineages (for details on the role of the Notch signaling pathway in endocrine versus exocrine specification, see previous section).

Acinar development is controlled by PTF1a, which, in association with RBP-J and RBP-L, promotes acinar differentiation and stimulates transcription of acinar-specific genes (Beres et al., 2006; Cockell et al., 1989; Fujikura et al., 2007; Jiang et al., 2008; Krapp et al., 1998; Masui et al., 2007). Mice deficient in PTF1a fail to form acinar cells (Krapp et al., 1998).

MIST1, a member of the B-class bHLH factors, is another transcription factor involved in acinar differentiation. During pancreas development, MIST1 is specifically expressed in acinar cells (Lemercier et al., 1997; Yoshida et al., 2001). It is required for acinar maturation and control of acinar cell proliferation (Jia et al., 2008; Pin et al., 2001; Rukstalis et al., 2003). However, the loss of MIST1 does not abrogate the transcription of most of the acinar-specific genes (Pin et al., 2001). Of the genes known to play a role in the pancreas, MIST1 is the only one completely restricted to acinar cells, and it can be hypothesized that MIST1 may be autoregulated to stabilize the acinar fate (Jensen, 2004). Very likely, PTF1a plays a role in the establishment of MIST1 expression, in a similar way to the NGN3 role in the NGN3/NeuroD cascade operating in endocrine development

There is also evidence that the Wnt/β-catenin pathway plays a role in acinar differentiation (Murtaugh et al., 2005), and acinar cell proliferation, mostly by stimulating expression of MYC, a factor required for acinar development (Bonal et al., 2009; Dessimoz et al., 2005; Heiser et al., 2006; Nakhai et al., 2008; Papadopoulou and Edlund, 2005; Strom et al., 2007; Wells et al., 2007).

1.3.4. Development of pancreatic ducts

Based on lineage tracing of PDX1-expressing cells, it has been shown that duct cells are derived from the same progenitor cells as the endocrine and acinar cells (Gu et al., 2002). The same study suggests that progenitors for endocrine and acinar cells, on one hand, and for duct cells, on the other hand, diverge around E12.5. Recently, it

has been demonstrated, that during pancreas development, the duct lineage segregates first from the acinar lineage and later from the endocrine lineage (Solar et al., 2009). This observation was made in lineage tracing experiments based on the expression of HNF1β, a transcription factor that is duct specific in the adult pancreas. The results indicate that HNF1β-positive cells present in the pancreas before E13.5 are precursors of acinar, duct, and endocrine cells. After E13.5, HNF1β-positive cells only gives rise to both duct and endocrine lineages, but not to acinar cells. By the end of gestation, the fate of HNF1β-positive cells is further restrained, as they only contribute to the duct lineage.

After an initial stage of lumen formation controlled by CDC42 and SDF1 (Hick et al., 2009; Kesavan et al., 2009), the duct network expands by branching morphogenesis (Puri and Hebrok, 2007). Compared to endocrine and acinar development, development of duct cells is poorly characterized at the molecular level. A few signalling pathways are important for branching morphogenesis. Inactivation of FGF10 or its receptor FGFr2IIIb leads to a reduced number of branches and decreased proliferation of the pancreatic progenitor cells (Bhushan et al., 2001; Pulkkinen et al., 2003). Secreted molecules of the epidermal growth factor (EGF) family are also important for duct growth and branching (Cras-Meneur et al., 2001; Miettinen et al., 2000). Permanent activation of Notch signaling allows the expression of several duct markers and represses endocrine and acinar fates (Hald et al., 2003b; Murtaugh et al., 2003b).

1.4. Role of HNF6 in pancreas development

Hepatocyte nuclear factor-6 (HNF6) is a liver-enriched transcription factor containing a bipartite DNA-binding domain composed of a single cut domain and a divergent homeodomain (Lannoy et al., 1998; Lemaigre et al., 1996). In the mouse

embryo, permanent or transient HNF6 expression has been detected in the liver, the intestinal epithelium, the nervous system, and the pancreas (Francius and Clotman, 2010; Landry et al., 1997; Rausa et al., 1997) . In the pancreas, HNF6 expression is initiated at E8.5 in the foregut endoderm and early pancreatic bud (Rausa et al., 1997). It is maintained in the developing pancreatic epithelium, until it becomes restricted to the duct cells in late gestation (Landry et al., 1997; Rausa et al., 1997).

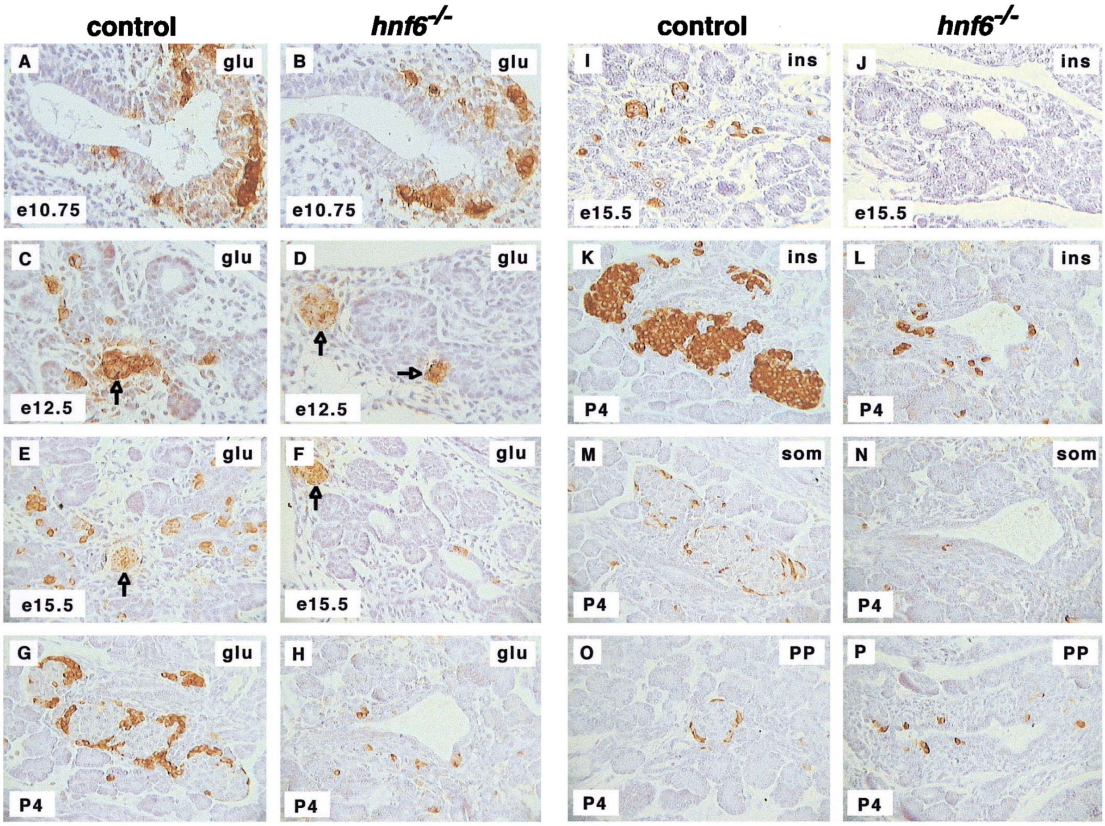

Figure 9. Defective endocrine cell differentiation and islet morphogenesis in hnf6-/- mice. *The number of glucagon (glu)-expressing cells is normal at E10.75 (A and B) but is reduced at E12.5, E15.5, and P4 (C-H). However, the number of clusters of early glucagon-expressing cells is normal at E12.5 and E15.5 (arrows in panels C-F). In hnf6-/- mice, there is a reduced number of insulin (ins)-expressing cells at E15.5 and four days after birth (P4) (I-L), of somatostatin (som)-expressing cells at P4 (M and N), and of PP-expressing cells at P4 (O and P). At P4, hormone-producing cells are found near pancreatic ducts in hnf6-/- mice, instead of being organized in islets as in control littermates (G, H, and K-P). From Jacquemin et al. (2000).*

HNF6 plays an important role in pancreas specification (Jacquemin et al., 2003). It binds to and activates the PDX1 gene promoter. In the absence of HNF6, PDX1 expression is delayed from the 11-somite stage to the 19-somite stage in the dorsal endoderm and from the 8-somite stage to the 22-somite stage in the ventral endoderm. The delay reduces the number of PDX1-expressing cells at the time when the pancreas buds out of the endoderm. This leads to hypoplasia of the buds and ultimately to hypoplasia of the entire pancreas at birth, despite a normalization of PDX1 expression levels from E10.75 to E17.5 (Jacquemin et al., 2000; Jacquemin et al., 2003).

HNF6 is also involved in pancreatic endocrine differentiation (Jacquemin et al., 2000). It binds to and stimulates the NGN3 gene promoter. In the absence of HNF6, the number of NGN3-expressing cells is drastically reduced and NGN3 mRNA levels are strongly decreased. As a consequence, endocrine differentiation is severely inhibited. In mice deficient for HNF6 (*hnf6-/-* mice), early glucagon expression is normal at E10.75. However, from E12.5, the number of glucagon-expressing cells is reduced by 85%. At E15.5, insulin-expressing cells are rare in *hnf6-/-* embryos. Four days after birth (P4), *hnf6-/-* animals show a markedly reduced number of α-, β-, δ-, and PP-cells (Figure 9). These four cell types are scattered within, and in the vicinity of pancreatic duct epithelium, instead of being clustered in islets as in control littermates. In *hnf6-/-* mice, the endocrine cells become organized in islets only 2 to 3 weeks after birth. The architecture of these islets is abnormal, as they show no mantle of α-cells around a core of β-cells, with many α-cells being located throughout the islets (Figure 10).

Besides its role in pancreas specification and endocrine differentiation, HNF6 has a role in the development of pancreatic ducts, where it is normally expressed at all developmental stages (Pierreux et al., 2006). In the absence of HNF6, the ducts develop cysts and are transiently devoid of primary cilia. This is associated with reduced expression of genes involved in polycystic diseases, namely those coding for HNF1β and for polyductin/fibrocystin and cystin (Pierreux et al., 2006). A requirement for normal function of primary cilia in duct development is further underscored by the finding that mice deficient in polaris, a ciliogenic protein, develop pancreatic cysts devoid of cilia (Cano et al., 2004).

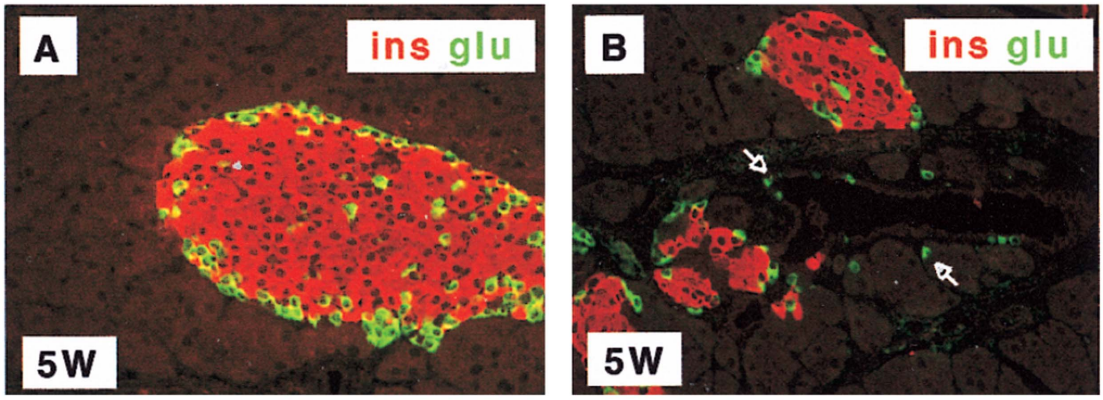

Figure 10. Formation of abnormal islets of Langerhans in hnf6-/- mice. *At 5 weeks (5W), islets of Langerhans were detected in hnf6-/- mice, but their architecture was perturbed and no mantle of glucagon (glu)-expressing cells (green) was seen around the islet core of insulin (ins)-expressing cells (red). From Jacquemin et al. (2000).*

Mice deficient in HNF6 are growth retarded at birth and show a reduced growth rate (Jacquemin et al., 2000). They have lower glucagonemia and insulinemia than wild type littermates, most probably as a result from the reduced α- and β-cell numbers. Newborn *hnf6-/-* are hypoglycemic, and 75% of them die between postnatal day 1 (P1) and P10, probably because of liver failure (cholestasis). The surviving mice reach adulthood but are diabetic. They have elevated fasting glycemia and are glucose intolerant. The perturbed glucose response is, at least in part, a consequence of insufficient glucose-induced insulin response, because insulin levels remain low after glucose injections. In addition, glucagonemia, measured after a fasting period, is slightly lower than in wild-type mice.

1.5 Production and regeneration of β-cells as a therapy for diabetes

A common denominator of all forms of diabetes is the lack of adequate functional β-cell mass (Donath, 2004; Donath and Halban, 2004; Rhodes, 2005; Rood et al., 2006). This lack, which is absolute in type 1 diabetes and relative in type 2 diabetes, causes insulin insufficiency leading to hyperglycemia (Figure 11).

Type 1 diabetes is characterized by auto-immune-mediated destruction of β-cells (Lenmark and Falorni, 1998), ending up in life-long dependence on insulin injections. In type 2 diabetes, risk factors like excessive caloric intake, obesity, or physical inactivity lead to insulin resistance. Under these circumstances, the β-cell mass expands and the islets of Langerhans grow in order to increase insulin release (Bruning et al., 1997; Butler et al., 2003; Kloppel et al., 1985; Kulkarni et al., 2004; Maclean and Ogilvie, 1955). Type 2 diabetes occurs when over time β-cell functionality decreases due to permanent overload, and the β-cell mass cannot keep up with the increase in peripheral insulin resistance (DeFronzo and Ferrannini, 1991; Kahn and Porte, 1996; Kruszynska and Olefsky, 1996; Rhodes, 2005).

Furthermore, people with type 2 diabetes may suffer from a slow-acting form of autoimmunity (LADA, latent autoimmune diabetes in adults or diabetes type 1.5), increasing the loss of β-cells and eventually leading to insulin dependency indistinguishable from type 1 diabetes (Tuomi et al., 1999; Zimmet et al., 1994).

Although insulin treatment has saved countless diabetics from early death, it represents an ameliorative treatment rather than a cure (Murtaugh, 2007). Modern therapies of blood sugar monitoring and insulin delivery cannot yet reach the precision of a functional β-cell, nor can they completely prevent the risk of debilitating long-term lesions caused by hyperglycemia.

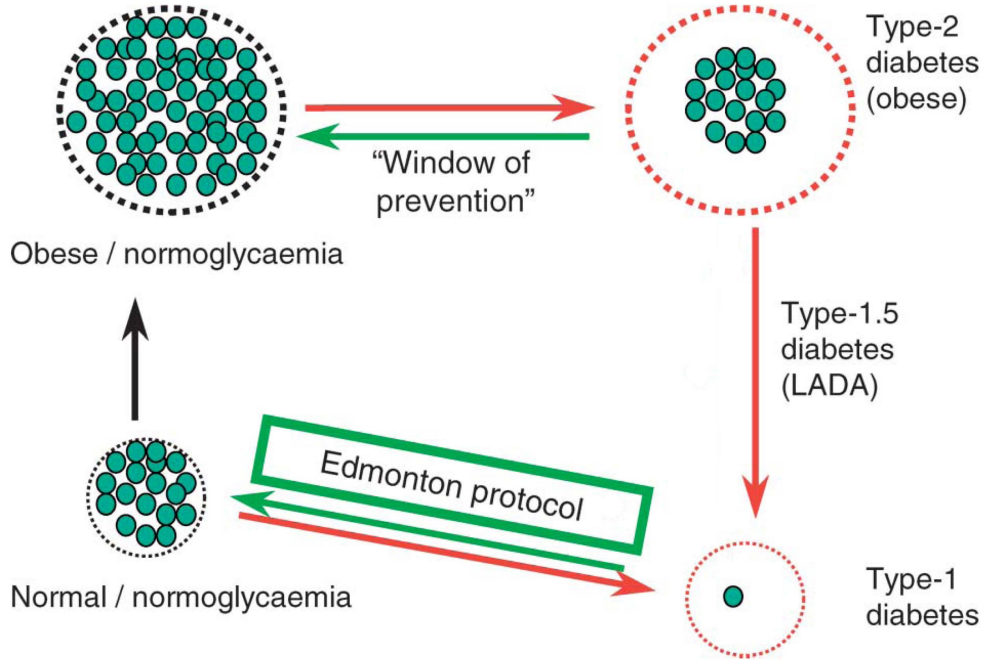

Figure 11. β-cell mass and diabetes. *The common denominator in all forms of diabetes is lack of functional β-cell mass and therefore, of insulin. There is an absolute versus a relative lack of β-cells in type 1 versus type 2 diabetes, respectively. A healthy islet of Langerhans is shown at the bottom left. The destruction of β-cells by the immune system leads to type 1 diabetes (bottom right). Insulin resistance induced by factors like overweight, increased caloric intake and no exercise, is compensated for by an increase in β-cell mass (top left). When this compensatory reaction fails to keep up with increasing insulin demands, type 2 diabetes ensues (upper right). When a slow immune-mediated process leads to further loss of β-cells in type 2 diabetic adults, this is designated as latent autoimmune diabetes in adults, LADA, or type 1.5 diabetes. Re-establishment of functional β-cell mass (green arrows) is expected to restore normalization of blood sugar regulation. The transplantation of organ donor islets of Langerhans into type 1 patients according to the Edmonton protocol constitutes a first step towards β-cell replacement therapies. Adapted from Madsen (2005).*

A true cure for diabetes might be achieved by replacing lost β-cells. Proof of principle that cell therapy of type 1 diabetes can potentially restore euglycemia was

provided by the "Edmonton protocol", which is based on transplantation of organ donor islets of Langerhans (Keymeulen, 2006; Keymeulen et al., 2006; Shapiro et al., 2000; Shapiro et al., 2006).

However, the limited availability of cadaver tissue means that transplants are only feasible for a small fraction of patients. It hence seemed obvious to try expanding β-cells in vitro for subsequent grafting. But even though replication of existing β-cells appears to be the major source of new β-cells in vivo (Dor et al., 2004; Georgia and Bhushan, 2004; Nir et al., 2007; Teta et al., 2007), such attempts have been hampered by the fact that in culture, adult β-cells are quite short lived and undergo dedifferentiation, with loss of insulin expression (Bar et al., 2008).

Hence, alternative approaches to generate β-cells for transplantation have been actively pursued, including differentiation of embryonic stem cells (ES cells), differentiation of adult stem cells, transdifferentiation of liver and of pancreatic cells to β-cells. In addition, several attempts have been made to stimulate the transdifferentiation of different pancreatic cell types to β-cells in vivo (Figure 12).

Differentiation of embryonic stem (ES) cells: They present two major advantages, namely their pluripotency and their high proliferation rate in culture. ES cells can be differentiated to insulin-producing cells by treatment with growth factors, according to protocols that mimic embryonic differentiation of β-cells. Until now, most ES cell-derived β-cells were not glucose responsive, either in vitro, or when grafted in vivo (Cho et al., 2008; D'Amour et al., 2006; Eshpeter et al., 2008; Jiang et al., 2007a; Mao et al., 2009; Phillips et al., 2007; Zhang et al., 2009). Exceptions are from a few protocols that could establish euglycemia when the cells were grafted in diabetic mice (Jiang et al., 2007b; Kroon et al., 2008; Shim et al., 2007). Even if the results are promising, several hurdles remain. The yield in endocrine cells is relatively low, and the heterogeneous nature of the resulting cell population is problematic, in particular with regard to potential contamination with undifferentiated ES cells susceptible to produce tumors. Moreover, the use of human ES cells raises ethical questions.

Differentiation of adult stem cells: It has been observed that the β-cell mass in adults is quite adaptive in response to altered metabolic demand, such as pregnancy or

obesity (Gupta et al., 2007; Karnik et al., 2007; Parsons et al., 1992). This has suggested that in addition to replication of existing β-cells, which is thought to be the major source of new β-cells after birth (Dor et al., 2004; Georgia and Bhushan, 2004; Nir et al., 2007; Teta et al., 2007), other cell types could be involved in β-cell regeneration and perhaps be stimulated in vivo to replenish the β-cell pool of diabetic patients. Multipotent stem cells were suggested to reside in pancreas (Seaberg et al., 2004; Suzuki et al., 2004). However, the data in this sense have never been confirmed. Bone marrow cells were also suggested to give rise to β-cells (Ianus et al., 2003; Moriscot et al., 2005; Sun et al., 2007), but these results have been questioned by others (Lechner et al., 2005; Taneera et al., 2006). Recently, grafts of bone marrow were not able to cure diabetes in humans (Esmatjes et al., 2010). Nevertheless, such protocols are used in "stem cell clinics" (Cell-Center at the Institute for Regenerative Medicine (2009) available from www.xcell-center.com, accessed 24 December 2009; Fundacion Don Roberto Fernandez Viña (2009) available from www.fundacionfernandezvina.org, accessed 24 December 2009), without strong scientific basis.

Transdifferentiation of liver cells: Protocols have been developed to transform liver cells to β-cells by forced expression of pancreas-specific transcription factors. Transfection of PDX1-expressing viruses combined with EGF/nicotinamide treatment can induce insulin production in cultured adult human liver cells (Sapir et al., 2005). When these cells were transplanted under the renal capsule of diabetic, immunodeficient mice, they reduced hyperglycemia. Nevertheless, strong skepticism remains whether manipulated liver cells, including virally infected cells, are safe enough for therapeutic use in humans.

Transdifferentiation of acinar cells: As far as the developmental origin is concerned, pancreatic acini and ducts are the cell types that have the most in common with β-cells. In vitro, acinar cells have the potential to express β-cell markers after treatment with EGF and leukaemia inhibitory factor (LIF) or EGF and nicotinamide (Baeyens et al., 2005; Minami et al., 2005). Inhibition of Notch signaling in rat acinar cultures promotes β-cell neogenesis, emphasizing the role of this pathway as an inhibitor of endocrine differentiation (Baeyens et al., 2009).

In vivo, the ability of acinar cells to change fate toward an endocrine lineage has been challenged by lineage tracing in different injury models. No contribution to either duct or endocrine lineage could be demonstrated (Desai et al., 2007). However, in vivo transdifferentiation of acinar cells to β-cells has been achieved by means of adenoviral transduction (Zhou et al., 2008). In this study, viruses designed to overexpress NGN3, MAFa and PDX1 were directly injected into the pancreas. This led to formation of insulin-expressing cells from acinar cells. The new cells expressed several β-cell specific genes including NeuroD, NKX2.2, and NKX6.1 and possessed morphologic characteristics of β-cells. When induced in diabetic mice, they significantly lowered the blood glucose level. However, they did not aggregate into islets, and it is not yet clear if these cells are completely functional β-cells.

Transdifferentiation of pancreatic duct cells: In vitro data suggest that pancreatic duct cells are able to give rise to insulin-producing cells (Bonner-Weir et al., 2000; Gao et al., 2003; Githens, 1988; Ramiya et al., 2000). Expression of islet hormones can be induced in duct cell lines upon exposure to exendin-4/GLP1, activin A, and HGF or betacellulin. Initiation of an endocrine program in cultured pancreatic duct cells can also be obtained by transfection with NGN-3-expressing adenoviruses (Gasa et al., 2004; Heremans et al., 2002).

After partial pancreatectomy, duct cells could transdifferentiate into β-cells, as suggested by the presence of insulin-positive cells in regenerating duct epithelium expressin PDX1 (Sharma et al., 1999). This suggestion has been supported by lineage tracing experiments using carbonic anhydrase (CA) II-CreER mice (Inada et al., 2008). The same study showed that duct cells transdifferentiate spontaneously into acinar and endocrine cells. However, misexpression of the lineage tracing construct in non-ductal cell types cannot be ruled out, as a human fragment of the CAII promoter was used to direct Cre expression (Puri and Hebrok, 2010). Also, this study claims that in wild type mice, 16.7% of the insulin-expressing cells derive from the ducts cells. This percentage was obtained with an efficiency of duct labelling of 30-50%. From these data, we deduced that the real percentage of β-cells deriving from duct cells should be around 33% - 56%, which is not in line with other lineage tracing studies claiming that β-cells are exclusively generated by proliferation of pre-existing β-cells (Dor et al., 2004; Georgia and Bhushan, 2004; Nir et al., 2007; Teta et al.,

2007). Moreover, another lineage tracing of duct cells using an HNF1β CreER mouse model, which labels duct cells in both embryonic and adult tissues, revealed no signs of duct-to-β-cell transdifferentiation (Solar et al., 2009). This study reported that duct cells do not contribute to the endocrine or acinar compartment under normal conditions or after injury. Moreover, after birth, or during β-cell growth triggered by ligation of the pancreatic duct or by cell-specific ablation with alloxan followed by EGF/gastrin treatment, the HNF1β-positive cells do not make a significant contribution to acinar or endocrine cells.

In an injury model of duct ligation, cells closely associated with the ductal tree and called facultative stem cells, are able to differentiate into islet cells (Xu et al., 2008). In this model, only the main duct leading to the tail of the pancreas and not the adjacent pancreatic parenchyma, was ligated. This led to acinar degeneration, duct expansion and induction of NGN3 expression.

Transdifferentiation of α-cells: Ectopic expression of PAX4 in α-cells converts them to functional β-cells (Collombat et al., 2009). The shift from α-cells to β-cells results in a glucagon deficiency that provokes a compensatory glucagon-cell neogenesis requiring the re-expression of the proendocrine gene NGN3.

In a recently published paper, it has even been demonstrated, that α-cells transdifferentiate spontaneously to β-cells, after a nearly total loss of β-cells (Thorel et al., 2010). This has been demonstrated in a transgenic mouse model of diphtheria-toxin-induced β-cell ablation combined with lineage tracing of α-cells. These findings point toward a high degree of plasticity inside the pancreatic endocrine compartment.

With regard to the therapeutic utility of β-cell production or regeneration, no protocol is yet appropriate for the treatment of diabetes. Low glucose response, questionable safety of grafted cells and of viral transduction, and ethical issues raised by the use of human embryonic stem still constitute major hurdles, indicating the need for more basic translational and applied research.

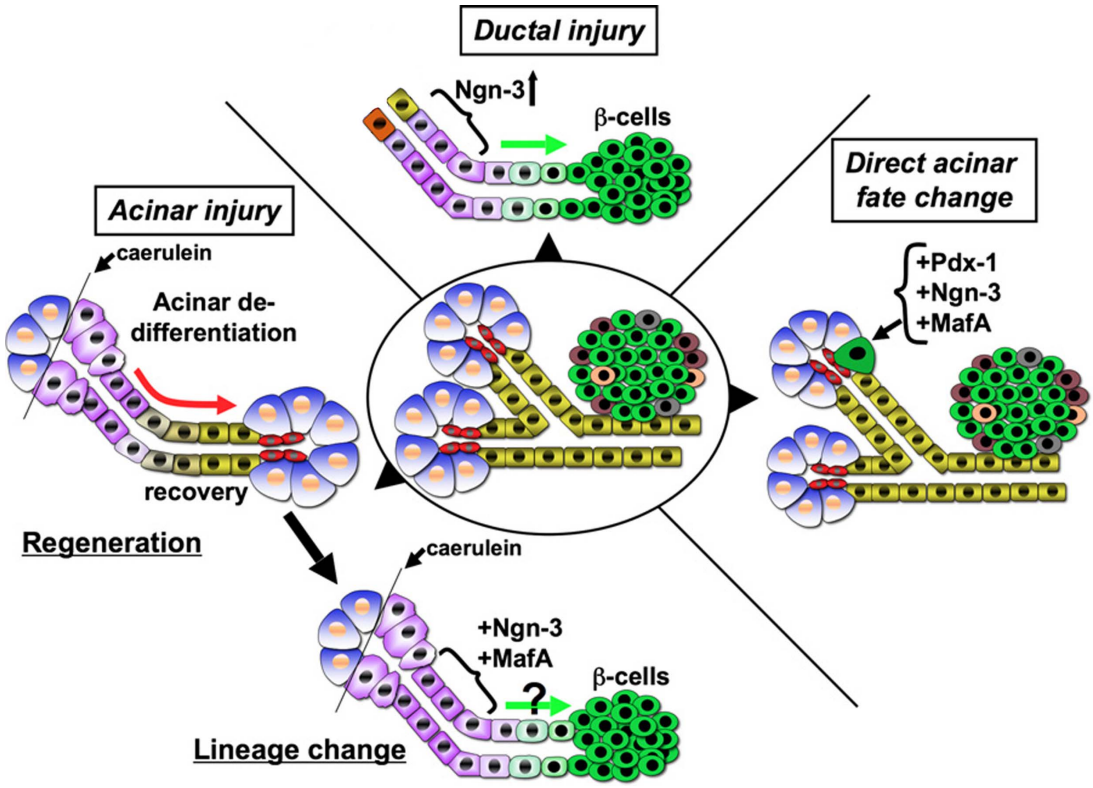

Figure 12. In vivo fate manipulation within the adult mouse pancreas. *In a ductal injury model (pancreatic duct ligation, top schematic), normal duct cells (yellow), or duct-associated cells (orange) adopt a progenitor state (purple) and upregulate NGN3 expression that correlates with increased β-cell formation, depicted by the green arrow. In an acinar cell injury model (caerulein treatment, left scheme), acinar cells (blue) undergo dedifferentiation (purple) followed by recovery (red arrow) and regeneration. Transfection of such dedifferentiated cells with key transcription factors like NGN3 and MAFa, might promote β-cell fate (bottom schematic). Another scenario is the direct acinar fate change (right panel). Transfection of adult pancreas with PDX1, NGN3, and MAFa redirects acinar cells to adopt a fate resembling endocrine β-cells (green). Adapted from Puri and Hebrok (2010).*

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2. Aims of the work

A promising way to cure diabetes is to implant in vitro produced β-cells, or to induce β-cell regeneration in vivo. Multiple attempts have been made to coax different cell types into β-cells. One of these cell types is the pancreatic duct cell, but its potential as a β-cell precursor remains unclear.

At birth, *hnf6-/-* mice have few β-cells and lack islets of Langerhans, but in adults the number of β-cells has increased and islets are present.

The aim of the present work is to analyze how β-cell neogenesis occurs after birth and to determine the origin of the newly formed β-cells.

To achieve this, we characterized the β–cell growth and performed lineage tracing of duct cells in *hnf6-/-* pancreata. The results show that neogenic β-cells derive from the pancreatic duct epithelium. These results are compiled in the second part of the "Results" section, the first part being focused on the optimization of a protocol for tracing the fate of pancreatic duct cells. A third part is devoted to the lineage tracing of pancreatic endocrine precursors in *hnf6-/-* mice.

3. Results

3.1. Optimized protocol for tracing the fate of pancreatic duct cells

3.1.1. Abstract

OBJECTIVE - Lineage tracing has become an essential tool to address the origin, the plasticity and the fate of cell types in normal and pathological conditions. Here we describe improvements in the lineage tracing of pancreatic duct cells, a cell type that is considered a potential source of β-cells for cell therapy of diabetes.

RESEARCH DESIGN AND METHODS - Protocols for tamoxifen administration were tested to optimize conditions for efficient labeling of pancreatic duct cells in embryos and postnatal mice that harbor a tamoxifen-inducible Cre recombinase driven by *hnf1β* gene regulatory regions (*hnf1β-creER*), and a *rosa26R* reporter gene.

RESULTS – For lineage tracing of pancreatic ducts in newborn pups, optimal results were obtained when mothers of litters received an intraperitoneal injection of 6 mg tamoxifen (dissolved in corn oil) combined with gavage of another 6 mg at the day of birth and repeated after 1 and 2 weeks, tamoxifen

being transferred to the pups via the milk. Tamoxifen treatment before sexual maturity led to sterility and growth retardation. In pregnant females, high doses of tamoxifen were compatible with embryo survival if tamoxifen was administered earlier than at e15.5.

CONCLUSIONS - Lineage tracing of pancreatic ducts in newborn pups is optimal by administration of 12 mg tamoxifen to the mother, partitioned in intraperitoneal injection and gavage. Repeated weekly tamoxifen administration improves the labeling efficiency. This protocol is expected to be also valid for lineage tracing of other cell types.

3.1.2. Introduction

Transgenic organisms designed to label and monitor a specific cell type over time, have become convenient tools to determine the origin, plasticity and fate of cells. The most popular lineage tracing techniques makes use of the *cre-loxP* (cyclization recombination-locus of crossing [x-ing]-over of bacteriophage P1) system (Branda and Dymecki, 2004; Feil et al., 2009). Constitutively active Cre or tamoxifen-inducible CreER excises a "floxed" stop transcription cassette located upstream of a reporter gene, typically inserted in the *rosa26* locus and whose activity can be monitored over time (Feil et al., 1996; Feil et al., 1997; Metzger et al., 1995; Soriano, 1999; Zhang et al., 1996).

For cell therapy of diabetes alternative sources of insulin-producing β-cells are being looked for (Borowiak and Melton, 2009), and transdifferentiated pancreatic duct cells are considered as potential candidates. Here we use *hnf1β-creER* mice for pancreatic duct cell lineage tracing (Solar et al., 2009). In postnatal pancreas, the transcription factor HNF1β is expressed in the cells lining the epithelium of main, interlobular, intralobular, and intercalated ducts, as well as in centroacinar cells. In the embryo it is expressed in primitive pancreatic ducts. In the *hnf1β-creER* model, Cre is specifically expressed in the duct cells in embryonic and adult pancreas (Solar et al., 2009). In the present work we describe an optimized protocol for pancreatic duct cell tracing.

3.1.3. Research design and methods

Mice and tamoxifen preparation/administration

hnf1β-creER mice (kind gift of J.Ferrer) were described (Solar et al., 2009). Pregnant mothers and mothers of litters were treated with tamoxifen (Sigma) dissolved in corn oil (Sigma) at 30 mg/ml. The solution was prepared under constant stirring at 37°C until complete dissolution of tamoxifen crystals. Tamoxifen was administered by intraperitoneal injection combined or not with gavage.

Detection of β-galactosidase activity

Samples were dissected in phosphate-buffered saline (PBS) and fixed for 20 min in fixation buffer (0.4% glutaraldehyde in phosphate buffer 100 mM, pH 7.4 with 2 mM $MgCl_2$ and 5 mM EGTA), then washed 3 times for 20 min in washing solution (0,01% Na-deoxycholate and 0,02% NP-40 in phosphate buffer 100 mM, pH 7.4 with 2mM $MgCl_2$). Samples were stained overnight at 30°C in 1 mg/ml X-Gal substrate (in the above described washing solution with 5mM K_3FeCN_6 and 5mM K_4FeCN_6).

Immunofluorescence

Tissue samples were fixed for 4.5 h (Cre/Mucin1 stainings) or 1.5 h (EYFP/Mucin1 staining) at 4°C in 4% para-formaldehyde in PBS, incubated overnight in PBS/20% sucrose and frozen in PBS/15% sucrose/7.5% gelatin. Frozen sections were cut to 10-12 µm on a cryostat. Sections were permeabilized in PBS/0.3% Triton X-100 and blocked with PBS/10%BSA/3% milk powder/0.3% Triton X-100 (for Cre/Mucin1 immunostainings) or with an antibody diluent containing background reducing components (DAKO)/3% normal donkey serum (Jackson) (for EYFP/Mucin1 immunostainings). Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C: armenian hamster anti-Mucin1 (NeoMarkers, 1:200), rabbit anti-Cre (kind gift from F.Tronche and C. Kellendonck, 1:3000), and rabbit anti-GFP (Abcam, 1:1000). Secondary antibodies (Alexafluor series from Invitrogen) were diluted in PBS/10%BSA/0.3% Triton X-100 (for Cre/Mucin1) or in the antibody diluent (DAKO)/3% normal donkey serum (for EYFP).

Ethics

All animal experiments were performed according to the institution's ethical guidelines.

3.1.4. Results and discussion

Toxicity of tamoxifen preparations were reported after oral administration of 1 mg, and the lethal dose is claimed to be 2 mg (Vasioukhin et al., 1999). Given our observations that toxicity of tamoxifen is increased when it is dissolved in ethanol, we dissolved tamoxifen in corn oil. We assessed the effect of increasing doses of tamoxifen by β-galactosidase (β-gal) labeling of pancreatic duct cells in newborn pups which can absorb tamoxifen provided to the mother through the milk (Leone et al., 2003). *hnf1β-creER:Rosa26R-lacZ* mice (Solar et al., 2009; Soriano, 1999) mothers were injected intraperitoneally at the day of birth with 30 mg/ml tamoxifen: 0.1, 0.5, 2 and 12 mg of tamoxifen were administered. The injected volume should remain below 200 µl to avoid peritonitis. Consequently, administration of 12 mg tamoxifen was split into an intraperitoneal injection of 200 µl (30 mg/ml) combined with the gavage of another 200 µl (30 mg/ml). 6 days after injection, pancreata were collected and stained to detect β-gal activity. At 0.1 mg, no cells positive for β-gal were observed in the pancreas (Figure 1A). Starting at 0.5 mg, the number of β-gal-positive cells increased gradually (Figure 1B-D). A 12 mg treatment gave the best labeling efficiency (Figure 1D). In these conditions, nuclear translocation of CreER 18 h after injection was observed in nearly 100% of duct cells, as revealed by co-labeling for Cre and the duct marker Mucin1 (Figure 1G-I). However, despite the efficiency of nuclear translocation of Cre, β-gal labeling efficiency was not maximal. Only few isolated cells were labeled in some ducts. Therefore, tamoxifen (12 mg as above) was administered to *hnf1b-creER:rosa26R-eYFP* mice mothers the day of birth and this was repeated after 1 and 2 weeks. With this protocol, long sections of ducts could be labeled (Figure 1K). However, some ducts were still unlabeled or only partially labeled (Figure 1L-M). A one-week delay between two administrations is required to avoid strong reduction in milk production by the mice.

The litters from mice that received 12 mg tamoxifen were growth retarded 7 days after injection (Figure 1J). At adulthood they caught up on size. Importantly, when pups were treated with tamoxifen they became sterile. However, fertility was unaffected in sexually mature mice treated with tamoxifen.

To optimize pancreatic duct cell tracing in embryos, pregnant mice at E14.5 were treated with 2 mg (intraperitoneal) or 12 mg (6 mg intraperitoneal + 6 mg gavage) of tamoxifen, and pancreata were collected at E17.5 and stained for β-gal activity. Labeling efficiency was higher with 12 mg treatment, as compared to 2 mg (Figure 1E-F). Administration of 12 mg tamoxifen to pregnant mice at E15.5 led to abortion.

In conclusion, the administration of 12 mg tamoxifen dissolved in corn oil and partitioned in intraperitoneal injections and gavage is optimal to trace the fate of pancreatic duct cells. Repeated tamoxifen administration improves the labeling efficiency.

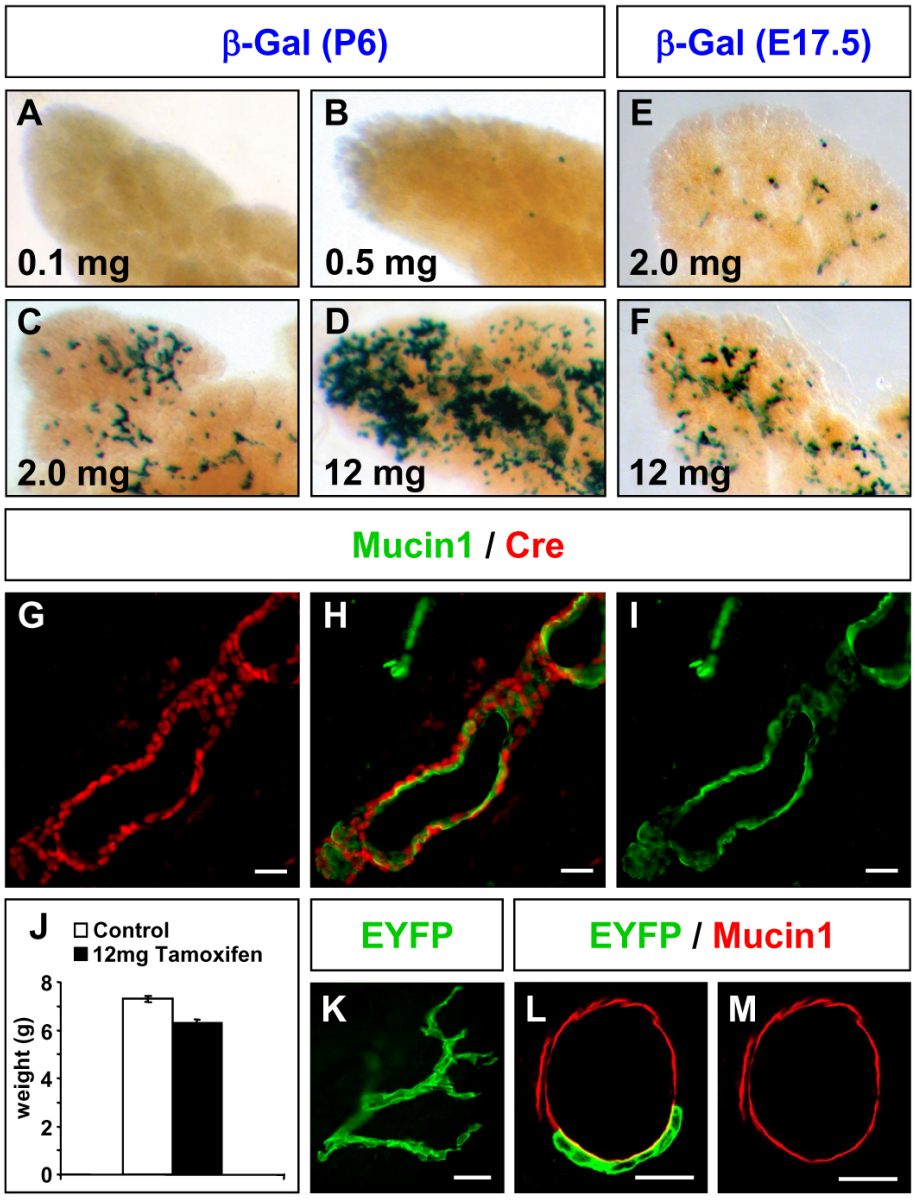

Figure 1. Effect of the dose of tamoxifen on the efficiency of pancreatic duct cell lineage tracing. *(A-D) β-gal expression in the pancreas of 6 day-old hnf1β-creER:rosa26R-lacZ reporter mice whose mothers were treated with tamoxifen at the day of birth. (E, F) β-gal expression in E17.5 hnf1β-creER:rosa26R-lacZ embryos whose mothers were treated with tamoxifen at E14.5. (G-H) Immunolabeling for Cre and the ductal marker Mucin1 in hnf1β-creER:rosa26R-eYFP pups 18h after administration of 12 mg tamoxifen to the mother. Scale bars, 20µm. (J) Weight of 7 day-old pups from litters whose mother was untreated or treated with 12 mg of tamoxifen at the day of birth. p<0.001, n=5 +/- SEM. (K-M) When mothers of hnf1β-creER :rosa26R-eYFP mice are treated at the day of birth with 12mg of tamoxifen as*

described above, with repeats after 1 and 2 weeks, long sections of ducts could be labeled (K). However, some ducts were still unlabeled or only partially labeled (L-M). The immunolabelings show staining for EYFP (K, L) and duct-specific Mucin1 (L, M). For this experiment, we used a hnf1β-creER:rosa26R-eYFP mouse line, which uses EYFP as reporter gene.

3.1.5. References

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3.2. Transdifferentiation of pancreatic duct cells to insulin-producing β–cells in the absence of the transcription factor HNF6

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Running title: HNF6 controls pancreatic duct cell plasticity

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3.2.1. Abstract

OBJECTIVE - A promising way to cure diabetes is to implant *in vitro* produced β-cells, or to induce *in vivo* β-cell regeneration. Multiple attempts have been made to coax different cell types into β-cells. One of these cell types is the pancreatic duct cell, but its potential as a β-cell precursor remains unclear. In mice deficient in Hepatocyte Nuclear Factor 6 (HNF6), a transcriptional regulator of pancreatic cell differentiation, the number of β-cells is reduced at birth, but subsequently undergoes a partial recovery. Here we examine the mechanism of β-cell recovery in *hnf6*-/- mice.

RESEARCH DESIGN AND METHODS – Immunodetection of differentiation markers, cell counts and mathematical models were used to characterize β-cell recovery in *hnf6*-/- mice. Genetic lineage tracing in *hnf6*-/-:*hnf1β-creER:rosa26R-eYFP* mice was performed to determine the origin of neogenic β-cells.

RESULTS - We found that β-cell neogenesis occurs in postnatal *hnf6*-/- mice. This results neither from compensatory proliferation of mature β-cells nor from delayed differentiation of β-cell precursors. Importantly, the presence of transition cells that co-express insulin and duct markers, and lineage tracing experiments indicate that duct cells transdifferentiate to β-cells in *hnf6*-/- pancreas.

CONCLUSIONS - Our results provide evidence that the absence of HNF6 promotes duct-to-β-cell transdifferentiation. This suggests new strategies for the production of β-cells from duct cells.

3.2.2. Introduction

The major defect in Type 1 diabetes is the reduction in the number of functional insulin-producing β-cells, leading to hyperglycemia. Despite therapy with insulin injections, the disease can cause severe and debilitating complications. Transplantation of

whole pancreas and grafting of isolated islets (Shapiro et al., 2000) can enable a cure for diabetes by replacing the deficient β-cells. However the shortage of donor pancreata restricts cell therapy to a minority of patients, thereby creating the need for new sources of insulin-producing cells (Borowiak and Melton, 2009).

During embryogenesis the β-cells are formed from precursor cells that express the transcription factor NGN3 (Gradwohl et al., 2000), and which differentiate perinatally into β-cells (Gu et al., 2002). Postnatally, in normal individuals, the β-cell population appears to be maintained by self-renewal (Dor et al., 2004; Teta et al., 2007). This has been demonstrated by studies that traced the fate of β-cells using chemical or genetic labeling methods, and showed that non-β-cells are unlikely to make a significant contribution to new β-cells after birth (Dor et al., 2004; Nir et al., 2007; Teta et al., 2007). Such studies are in contradiction with a lineage tracing experiment proposing that a major fraction of new β-cells formed postnatally are derived from pancreatic duct cells (Inada et al., 2008). However, a more recent lineage tracing study using a hormone-inducible Cre recombinase that labels the differentiated pancreatic duct epithelium shows that postnatal duct cells retain the capacity to self-renew, but do not give rise to acinar or β-cells (Solar et al., 2009).

Production of β-cells by other means than self-renewal has been reported following overexpression of transcription factors in exocrine cells (Zhou et al., 2008), pancreas injury (Xu et al., 2008), or forced expression of a transcription factor in other pancreatic endocrine cells (Collombat et al., 2009). In the latter two studies, the existence of facultative β-cell progenitors has been proposed. However, until now, the origin of such progenitors is still unknown. Therefore, our understanding of which cell types can provide a source of new β-cells remains limited. In the present work, we characterize β-cell neogenesis (here defined as the postnatal formation of β-cells) in the pancreas of mice deficient in HNF6, and show that duct cells in these mice give rise to β-cells.

3.2.3. Research design and methods

Mice

hnf6-/- and *hnf1β-creER* reporter mice have been described (Jacquemin et al., 2000; Solar et al., 2009). *hnf1β-creER* reporter mice were first crossed with *rosa26R-eYFP* (Srinivas et al., 2001)*:hnf6+/-* mice. The resulting *hnf1β-creER:rosa26R-eYFP:hnf6+/-* mice were then crossed with *hnf6+/-* mice to produce control and *hnf6-/-* mice in the *hnf1β-creER:rosa26R-eYFP* background. Mothers of these litters were treated with tamoxifen (Sigma) dissolved in corn oil (Sigma) at 30 mg/ml. Treatment consisted of an intraperitoneal injection of 200 µl combined with the gavage of another 200 µl at the day of birth with repeats after 1 and 2 weeks. All animal experiments were performed according to the institution's ethical guidelines.

Immunohistochemistry and immunofluorescence

Tissue samples were fixed overnight at 4°C in 4% para-formaldehyde dissolved in phosphate-buffered saline (PBS). After paraffin-embedding, sections were cut to 7 µm. Heat-mediated antigen retrieval was performed in citrate buffer (pH 6) either in a microwave oven or at 99°C in a PT Module (Labvision). All samples were permeabilized in PBS/0.3% Triton X-100 and blocked with PBS/10%BSA/3% milk powder/0.3% Triton X-100. The following primary antibodies were diluted in blocking buffer and incubated overnight at 4°C: guinea pig anti-insulin (DAKO, 1:1000), rabbit anti-PDX1 (gift from C.Wright, 1:2000), rabbit phosphohistone H3 (Cell Signaling, 1:50), rabbit anti-NGN3 (gift from M.German, 1:500), rabbit anti-HB9 (Abcam, 1:3000), rat anti-CK19 (DSHB, 1:50), rabbit anti-SOX9 (Chemicon, 1:250), rabbit anti-HNF1β (Santa Cruz, 1:50), armenian hamster anti-mucin1 (NeoMarkers, 1:200), rabbit anti-Cre (gift from F.Tronche and C. Kellendonck, 1:3000), rabbit anti-GFP (Abcam, 1:1000), goat anti-GFP (Abcam, 1:100), mouse anti-E-Cadherin (BD, 1:1000). Secondary antibodies (Alexafluor series from Invitrogen) were diluted in PBS/10%BSA/0.3% Triton X-100. For SOX9 immunostaining, a TSA signal amplification (Invitrogen) step was added. For Cre immunostainings, samples were incubated overnight in PBS/20% sucrose and frozen in PBS/15% sucrose/7.5% gelatin. The anti-Cre antibody required no antigen retrieval.

Frozen sections were cut to 10 µm on a cryostat. For the anti-EYFP staining with rabbit anti-GFP (Abcam), we used Tris buffer (pH 10) at 60°C.

Ethics

All animal experiments were performed according to the institution's ethical guidelines.

3.2.4. Results

Compensatory growth of β-cells in mice deficient in HNF6

During pancreas development, expression of HNF6, a transcription factor of the Onecut family (Jacquemin et al., 2003b; Lannoy et al., 1998; Lemaigre et al., 1996; Vanhorenbeeck et al., 2002) is first detected in the pancreatic progenitor cells and then becomes restricted to the duct epithelium in late embryogenesis (Jacquemin et al., 2003a; Pierreux et al., 2006). HNF6 is required for the generation of the NGN3-expressing endocrine precursor cells (Jacquemin et al., 2000). Consequently, in newborn *hnf6-/-* mice, the number of β-cells is markedly reduced and no islets of Langerhans are formed. Interestingly, in adult *hnf6-/-* mice the number of β-cells increases and islets are observed (Jacquemin et al., 2000), thus suggesting the existence of β-cell neogenesis. To characterize this process, we first quantified the number of β-cells in *hnf6-/-* pancreas at different stages and compared it to wild-type pancreas (Supplemental Methods I). The relative deficit in β-cells in *hnf6-/-* mice decreased with time (Figure 1*A*): at embryonic day 15.5 (e15.5), we found a 31-fold reduction in β-cell number in *hnf6-/-* pancreas as compared to control pancreas. However, this difference was reduced to 10-fold at e17.5, to 9.4-fold at postnatal day 4 (4d), and to 5-fold three weeks (3w) after birth (Figure 1*A*). This confirmed that a compensatory growth of β-cells takes place in *hnf6-/-* mice.

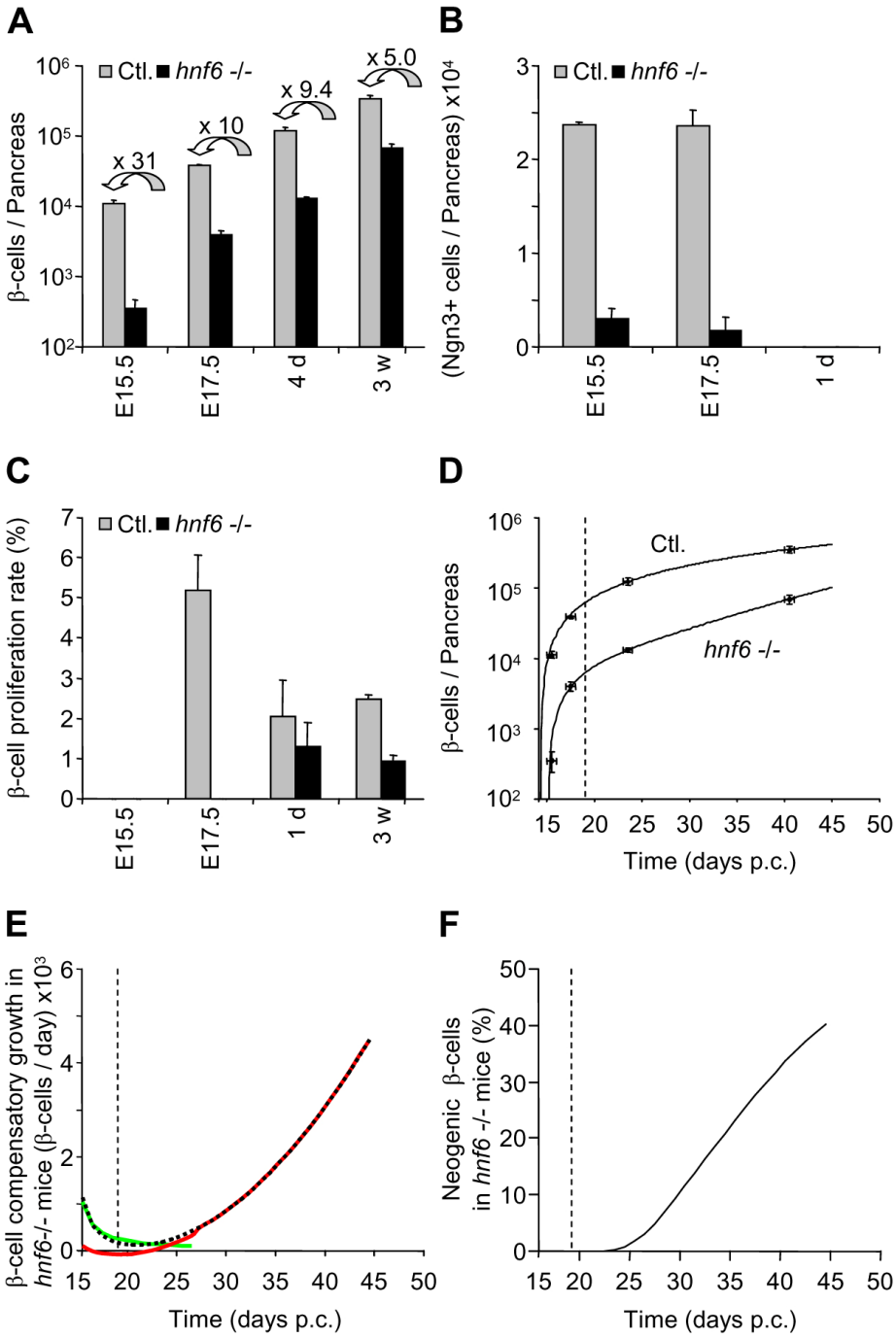

Figure 1. β-cell neogenesis in hnf6-/- mice*. A-C: Quantification of β-cells (A), NGN3+ precursor cells (B), and β-cell proliferation (C) at different stages in control (ctl) and hnf6-/- mice shows that the relative β-cell deficit in hnf6-/- mice decreases with time, and that the compensatory growth does not depend on delayed differentiation of NGN3+ precursors or on enhanced β-cell proliferation. The β–cell proliferation rate is defined as the proportion of β-cells positive for the proliferation marker phosphohistone H3.*

D-F: Mathematical modeling of β-cell development suggests the existence of postnatal β-cell neogenesis in hnf6-/- mice. (D) Total β-cell numbers in ctl and hnf6-/- mice. (E) Modeling of β-cell compensatory growth in hnf6-/- mice. The dotted black curve indicates the number of β-cells produced each day in hnf6-/- mice due to an increase in relative speed of β-cell generation over control mice. The green curve represents the contribution of NGN3+ cells in hnf6-/- mice, and the red curve the neogenic β-cells (i.e. the difference between the dotted black and the green curve). (F) Percentage of neogenic β-cells in hnf6-/- mice. Error bars represent s.e.m., n = 3. The horizontal axis in D to F corresponds to days post coitum (days p.c.). The dashed vertical lines correspond to the day of birth.

β-cell neogenesis is present in *hnf6-/-* mice and does not involve endocrine precursors or increased proliferation of β-cells

To assess if the compensatory growth results from a delay of the normal process of embryonic formation of pancreatic endocrine cells, we quantified NGN3-expressing cells at different stages (using the same method as in Supplemental Methods I). We found no evidence for delayed differentiation, since at e15.5 and e17.5, *hnf6-/-* pancreata contained 7.8 and 13.8 times less endocrine precursors than normal pancreata, respectively (Figure 1*B*), whereas between postnatal day 1 and 3 weeks of age, no NGN3-positive cell (NGN3+) could be detected by immunostainings in control or *hnf6-/-* pancreata (Figure 1*B*). NGN3 expression was detected after birth by Q-PCR but levels were very low and similar in control and *hnf6-/-* pancreata (Supplemental Figure 1).

We then asked if a higher proliferation rate of the β-cells in *hnf6-/-* mice could account for the compensatory β-cell growth. This was again not the case, because at all tested stages proliferation of β-cells was lower in *hnf6-/-* mice than in control mice (Figure 1*C*). The β–cell proliferation rate is here defined as the proportion of β-cells positive for the proliferation marker phosphohistone H3, which is characteristic of mitotic cells. The proliferation of NGN3+ endocrine precursors at e15.5 was also measured but no significant difference between *hnf6-/-* and control pancreata was detected. Also, apoptosis was tested by TUNEL-assays and immunolabeling of caspase3. Apoptosis was barely detectable in control and *hnf6-/-* mice (data not shown).

To analyze the kinetics of β-cell generation in control and *hnf6-/-* mice, we developed a mathematical model based on the cell counts (for details, see Supplemental Methods II). We took the data from Figure 1*A* and defined the best fitting trend line for each dataset

(Figure 1*D*). The corresponding equations allowed us to predict the absolute number of β-cells present in control and *hnf6-/-* at any given developmental stage. We used this to calculate the relative speed of β-cell generation in *hnf6-/-* and control pancreata. We quantified the difference between *hnf6-/-* and control pancreata (Figure 1*E*, dotted black curve), and calculated the contribution of new β-cells derived from embryonic NGN3+ endocrine precursors (Figure 1*E*, green curve; see Supplemental Methods II for the calculation of endocrine precursor contribution). We then estimated the compensatory β-cell growth that cannot be accounted for by NGN3+ cell-derived cells (red curve). This analysis indicated that the contribution of NGN3+ endocrine precursors accounts for the compensatory growth observed before birth, and that a different process takes place after birth. The latter process– which we call β-cell neogenesis- accounts for a production of ~3000 β-cells/day at 3 weeks after birth. We calculated that β-cell neogenesis contributes to at least 1/3 of all β-cells found in *hnf6-/-* pancreas at 3 weeks after birth (Figure 1*F*).

Differentiating β-cells are found in or near the duct epithelium of *hnf6-/-* mice

Next, we tried to elucidate the origin of the neogenic β-cells in *hnf6-/-* mice. The low proliferation rate of β-cells in *hnf6-/-* mice (Figure 1*E*), and the earlier observation that many β-cells in these mice are located in the vicinity of the pancreatic ducts (Jacquemin et al., 2000), suggest that transdifferentiation of duct cells might contribute to β-cell neogenesis. This suggestion is supported by the observation that postnatal *hnf6-/-* pancreata contain HB9+/insulin- cells, which are β-cell precursors normally found in the pancreatic epithelium of wild-type mice during embryogenesis, but not postnatally (Figure 2*A-C*). In *hnf6-/-* mice, these HB9+/insulin- cells are located within the duct epithelium, in areas where it is multilayered or lining cysts. These findings suggested that adult duct cells in *hnf6-/-* mice might recapitulate in part the embryonic differentiation pathway.

To assess if neogenic β-cells in *hnf6-/-* mice derive from duct cells, we first sought to identify transition cells that co-express insulin and duct markers. We thus performed immunostainings for insulin and two duct-specific markers: the cytoskeleton protein cytokeratin 19 (CK19) (Brembeck et al., 2001; Deramaudt et al., 2006), and the transcription factor SOX9 (Seymour et al., 2007). Control islets and ducts, as well as *hnf6-/-* islets, do not show coexpression of insulin and CK19 (Ins+/CK19+) (Figure 2*D* and *E*), except for very rare cells in the islets (not shown). However, in *hnf6-/-* duct epithelium, numerous Ins+/CK19+ cells were detected (Figure 2*F*). In addition, insulin+/SOX9+ cells

were found in *hnf6-/-* ducts (Figure 2*G-I*). These results are consistent with a duct-β-cell transition state.

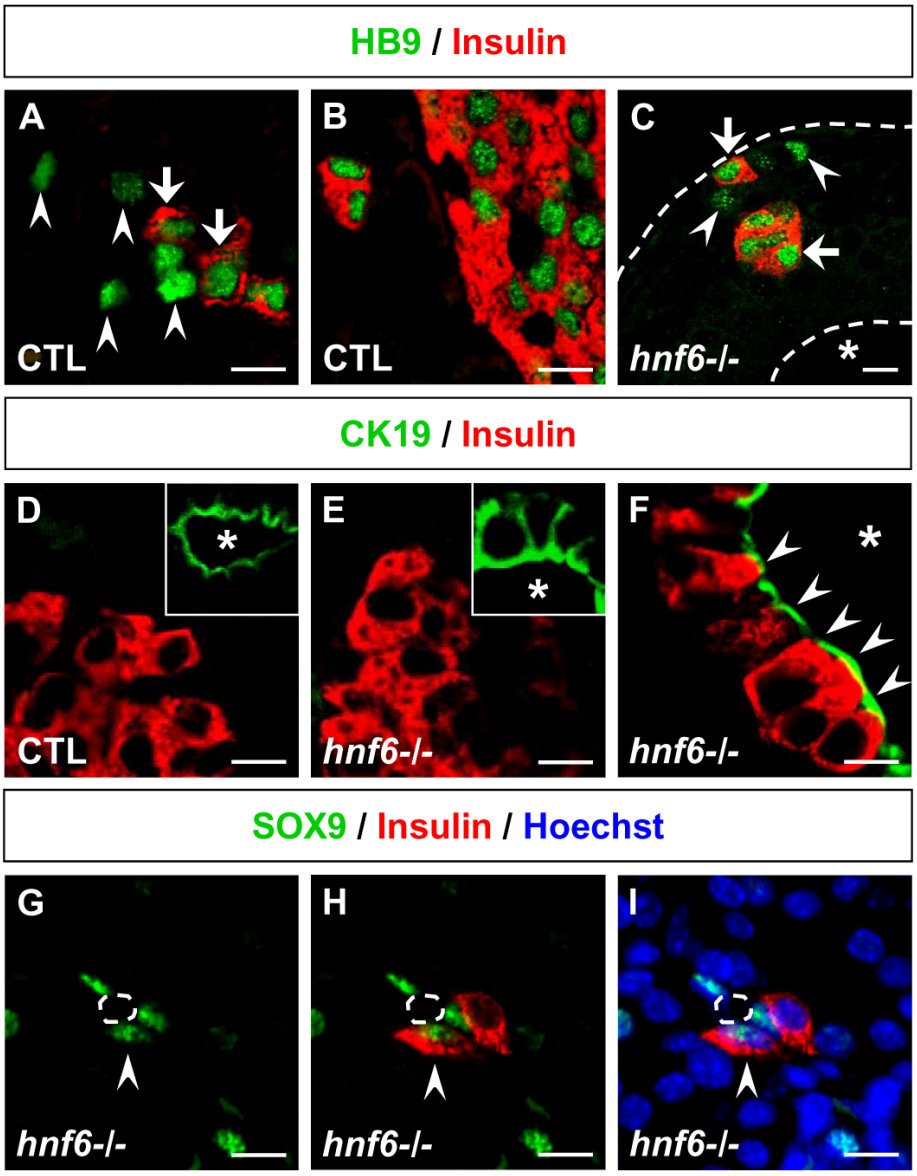

Figure 2. Expression of differentiation markers in HNF6-deficient duct and insulin-expressing cells. *A-C: In control mice at E15.5, HB9 is expressed in the β-cell precursors (A), whereas one week after birth HB9 is found exclusively in insulin-producing cells (B). In hnf6-/- mice, HB9+/insulin- cells are found in the duct epithelium (C). Arrowheads point to cells expressing HB9 but not insulin; arrows point to β-cells expressing both HB9 and insulin. Dotted lines in panel C delineate the duct epithelium, which is multilayered, or lining cysts as a result of the absence of HNF6. D-F: In 1-week-old wild-type mice the expression of CK19 and insulin does not overlap and marks duct cells and β-cells, respectively (D). In hnf6-/- mice at the same stage, islets cells are insulin+/CK19- (E), but cells co-expressing both markers are found in the duct epithelium, suggesting the existence*

of a duct-to-β-cell transition state (F). Arrowheads point to co-expression of CK19 and insulin; Insets contain ducts stained with CK19. Stars refer to the duct or cyst lumen. G-I: Rare cells co-expressing insulin and the duct marker SOX9 and can be found in 3-week-old hnf6-/- mice, further suggesting the existence of transition cells (arrowheads). Dotted lines delineate a duct. Scale bars, 10µm.

Expression of duct-specific markers is reduced in *hnf6-/-* pancreata

A number of duct cells in *hnf6-/-* cells showed reduced expression of SOX9 and HNF1β, a transcription factor that is also specifically expressed in the duct epithelium (Solar et al., 2009). Whereas in control pancreas all cells lining the ducts expressed both SOX9 and HNF1β (Figure 3*A* and *C*), in the epithelium that lines ducts of *hnf6-/-* mice, cells expressing both factors are intermingled with cells that do not express SOX9 and/or HNF1β (Figure 3*B* and *D*). However, the latter still express mucin-1, and therefore have duct characteristics (Figure 3*F* and *H*). These results suggest that *hnf6-/-* duct cells progressively lose the expression of specific markers, before gaining the ability to differentiate into β-cells.

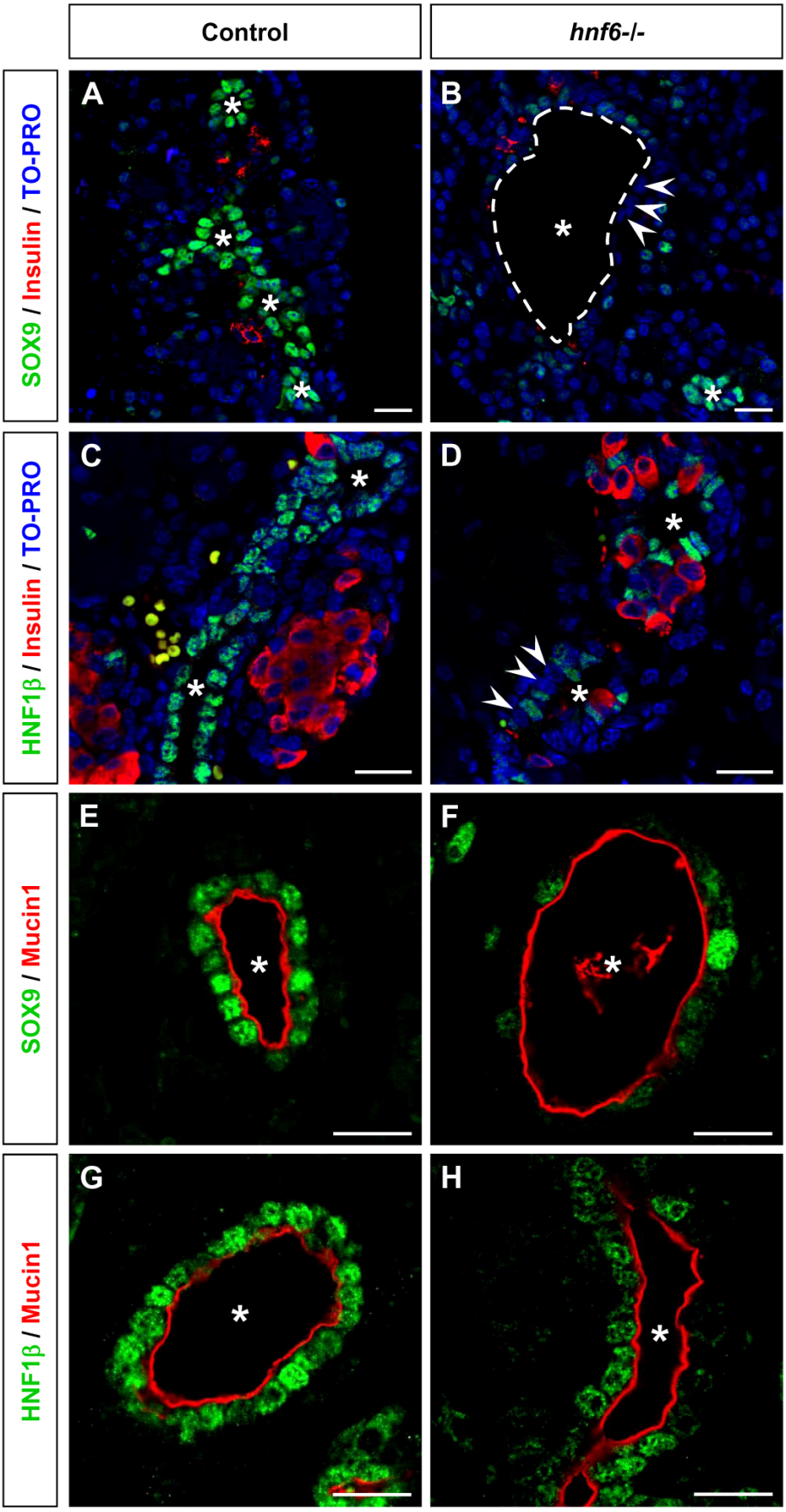
Control
hnf6-/-
SOX9 / Insulin / TO-PRO
HNF1β / Insulin / TO-PRO
SOX9 / Mucin1
HNF1β / Mucin1
A
B
C
D
E
F
G
H

Figure 3. Reduced expression of duct markers in hnf6-/- duct epithelium*. A-D: The expression of SOX9 in 1-day-old mice (A and B) and of HNF1β at E17.5 (C and D) is reduced in hnf6-/- mice as compared to controls. E-H: Despite a reduction in the expression of SOX9 and HNF1β in 1-week-old hnf6-/- mice, all duct cells maintain the expression of mucin1 (F and H), as compared to control mice (E and G). The lumen of ducts and cysts is indicated by stars or delineated by dotted lines. The arrowheads point to duct cells that do not express SOX9 or HNF1β in hnf6-/- pancreas. Scale bars, 20µm.*

Lineage tracing experiments indicate that duct cells transdifferentiate to β-cells in *hnf6-/-* mice

To directly test the hypothesis of duct-to-β-cell transdifferentiation in *hnf6-/-* mice, we performed lineage tracing experiments in which duct cells and their progeny were labeled. We used a BAC transgenic mouse strain in which expression of a tamoxifen-inducible Cre recombinase (CreER) was controlled by the regulatory regions of the transcription factor HNF1β (Solar et al., 2009). Recent studies using this model showed that HNF1β-expressing duct cells of the embryonic pancreas give rise to endocrine cells, whereas after e18.5 the duct epithelium no longer contributes to the formation of endocrine cells (Solar et al., 2009). In our experiments, we treated litters resulting from crossings between *hnf1β-creER:rosa26R-eYFP:hnf6+/-* and *hnf6+/-* mice with tamoxifen from the day of birth until 2 weeks after birth. Sixteen hours after tamoxifen treatment the expression of Cre recombinase was nuclear and detectable only in duct cells. This indicated that the expression of Cre faithfully recapitulated that of HNF1β, and thus validated the duct specificity of the model in control (Figure 4*A-C*) and *hnf6-/-* (Figure 4*D-F*) mice. Co-labeling experiments with insulin and EYFP were then performed in tamoxifen-treated 5 week-old mice to test if any β-cells were derived from duct cells. Consistent with previous findings (Solar et al., 2009), in control mice, EYFP expression was highly duct-specific (Figure 4*G-H*), with a maximum labeling efficiency of 50 %. Thus, no Ins+/EYFP+ cells were detected (Figure 4*I*). In contrast, Ins+/EYFP+ cells were detected in *hnf6-/-* mice. These Ins+/EYFP+ cells were located outside the duct epithelium (Figure 4*J-L*) or formed buds from the epithelium (Figure 4*M-R*). These findings therefore indicate that insulin-expressing cells can originate from duct cells in mice lacking HNF6, thus providing a mechanism for the postnatal compensatory β-cell growth observed in this model.

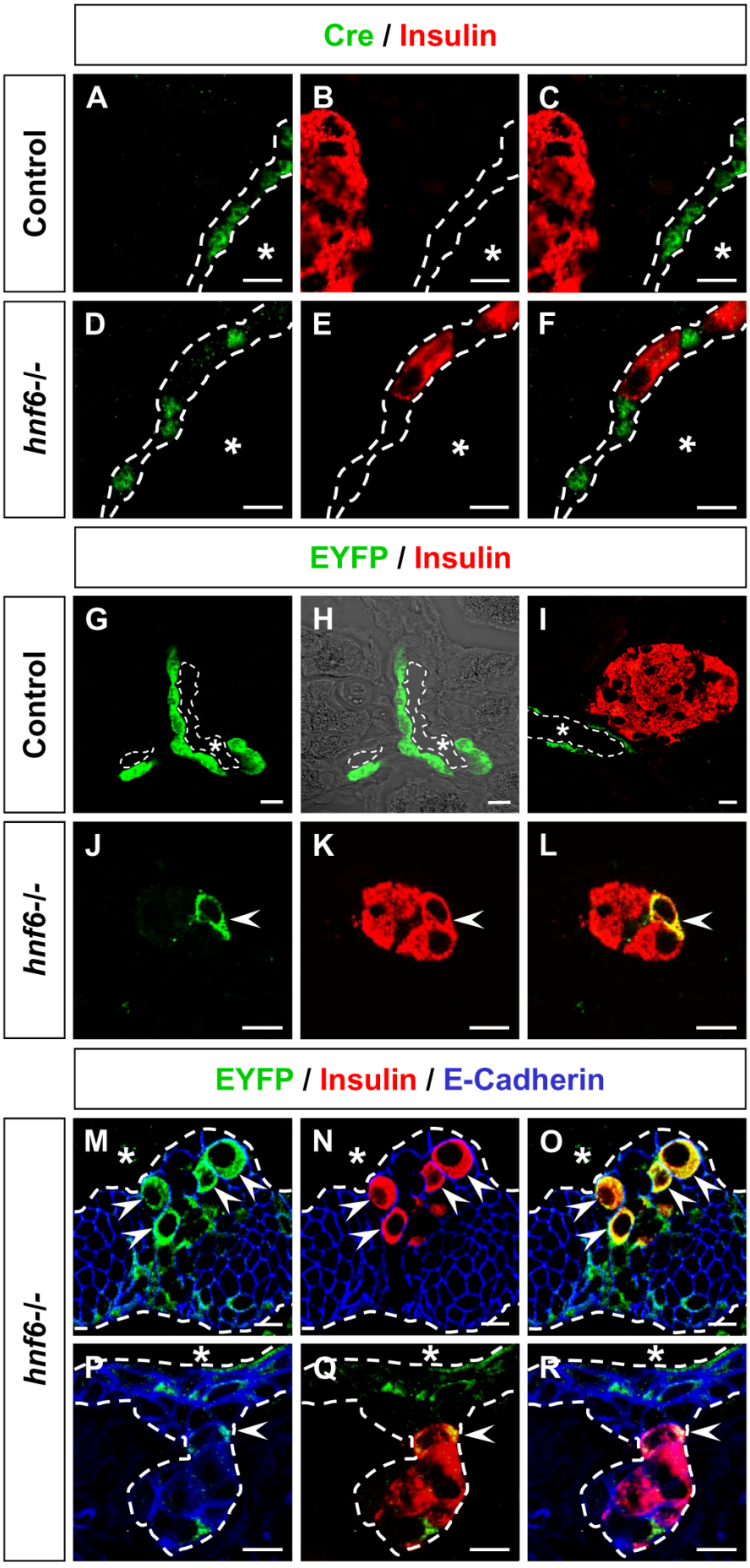
Cre / Insulin
Control
hnf6-/-
A
B
C
D
E
F
EYFP / Insulin
Control
hnf6-/-
G
H
I
J
K
L
EYFP / Insulin / E-Cadherin
hnf6-/-
M
N
O
P
Q
R

Figure 4. Lineage tracing of duct cells in control and hnf6-/- mice. *A-F: The expression of Cre recombinase is restricted to duct cells in 5-week-old control (A-C) and hnf6-/- mice (D-F), 16h after tamoxifen injection. The lumina of ducts are indicated by a star and the epithelium lining ducts is surrounded by a dotted line. G-R: EYFP expression induced by Cre recombinase activity is restricted to duct cells in hnf1β-creER:rosa26R-eYFP mice (G-I; in panel H, phase contrast is superimposed to fluorescent staining), 3 weeks after the last tamoxifen injection. In contrast, co-expression of EYFP and insulin (arrowheads) is found in hnf1β-creER:rosa26R-eYFP:hnf6-/- mice (J-R). Such EYFP+/insulin+ cells are found outside the epithelium (J-L) or budding from the epithelium (stained for E-cadherin and surrounded by dotted lines; M-R). Stars refer to the lumen of ducts. Scale bars, 10 µm.*

3.2.5. Discussion

hnf6-/- mice, which have deficient differentiation of endocrine cells, show partial recovery of β-cell numbers after birth. By analyzing the differentiation of regenerating β-cells and using lineage tracing experiments, we show in the present paper that β-cell recovery depends on transdifferentiation of pancreatic duct cells.

A number of studies addressed the mechanism of β-cell regeneration in various animal models, and the origin of the regenerating cells is intensely debated (Kushner et al., 2010). Recent lineage tracings indicate that wild-type duct cells do not contribute to β-cell development in normal conditions, after pancreatic duct ligation or after alloxan-mediated β-cell ablation (Solar et al., 2009). In contrast, using the same lineage tracer mice, we now provide evidence that duct cells can contribute to β-cell regeneration, in the absence of the transcription factor HNF6.

HNF6 is required for the expression of duct-specific genes in the embryonic pancreas (Pierreux et al., 2006). We now show that after birth, the *hnf6-/-* duct cells express typical duct markers such as SOX9, HNF1β, CK19 or mucin1, but the expression level of these differentiation markers is not equal in all cells. This indicates that the duct epithelium is heterogeneous and raises the question of the characteristics of the *hnf6-/-* duct cells able to give rise to insulin-producing cells. The *hnf6-/-* duct epithelium may contain bipotent progenitors, similar to those present in the embryonic pancreas until e16.5 and which can give rise to duct and endocrine cells (Solar et al., 2009). However, such embryonic

progenitors differentiate to β-cells by transiting through an endocrine precursor state, characterized by the expression of high levels of NGN3. Since such NGN3+ cells were not found in postnatal *hnf6-/-* pancreas, the duct cells which give rise to insulin-producing cells after birth are unlikely to be similar to bipotent duct progenitors found in embryos. Postnatal duct cells in wild-type animals can only self renew (Solar et al., 2009). In contrast, *hnf6-/-* duct cells can differentiate to β-cells, suggesting that the lack of HNF6 confers or restores an endocrine differentiation potential. In that case, the absence of HNF6 would promote the differentiation plasticity of the duct cells. Consequently, it is now important to understand how HNF6 controls the duct differentiation potential. Characterizing such control mechanisms should contribute to the design of strategies to coax duct cells into β-cells.

Our data also suggest that cell reprogramming in the pancreas can be obtained not only by overexpressing transcription factors (Collombat et al., 2009; Zhou et al., 2008), but also by inhibiting the expression of factors important for acquiring or maintaining a differentiated state. A similar concept is operational in the hematopoietic system where B lymphocytes lacking PAX5 give rise to T lymphocytes (Cobaleda et al., 2007). In our study, duct-to-β-cell transdifferentiation is restricted to a subset of duct cells, indicating that yet unidentified factors are involved in this process in parallel to the absence of HNF6. Manipulating the expression of such factors coupled with inhibition of HNF6 is expected to increase the efficiency of the duct-to-β-cell transdifferentiation. This opens avenues in the search for new sources of β-cells for treating diabetes.

3.2.6. Acknowledgements

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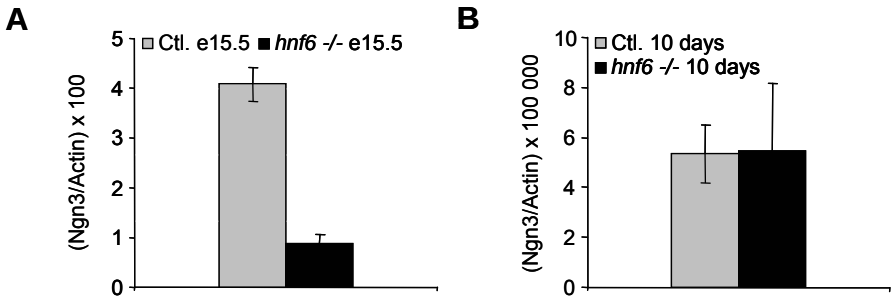
3.2.7. Supplemental methods and figures



Supplemental Figure 1. Q-PCR for NGN3 shows that expression levels of NGN3 decrease strongly after birth in control and hnf6-/-mice. (*A-B): Q-PCR for NGN3 has been performed on e15.5 control (n=8) and hnf6-/- (n=5) pancreas (A) and on pancreas of 10 day old control (n=4) and hnf6-/- (n=3) mice (B). Pancreata were isolated on ice-cold phosphate-buffered saline containing RNAsin (RNAsin Ribonuclease Inhibitor, Promega, 40U/µl). Total RNA was extracted with Trizol RNA Isolation reagent (Invitrogen). Reverse transcription and quantitative real-time polymerase chain reaction (PCR) of total RNA were performed as described (Pierreux et al., 2006). Samples without reverse transcripase were used as negative controls. Threshold cycles were transformed to copy number according to the standard calibration curve. Absolute copy number for NGN3 messenger RNA (mRNA) was normalized to absolute actin mRNA copy number. Results are presented in Supplemental Figure 1A and B. Primer sequences were 5'-TCACTGACTGACCTGCTGCT-3' and 5'-TTGGAACTGAGCACTTCGTG-3' for NGN3. Primer sequences for actin were as described (Pierreux et al., 2006).*

Supplemental methods I:

Quantification of total β-cell numbers

To quantify the total number of β-cells, whole pancreata were cut in 7 µm sections. 16 equally spaced sections were selected: every N/17 (N = total number of sections obtained from a pancreas) section was mounted for immunofluorescence. Insulin/PDX1 immunostaining was performed as described in *Methods*. The total number of β-cells was counted on each section. The counting results were plotted as a function of the position of each section in the pancreas (Figure SM1 shows an example of a control pancreas at E17.5). The surface under the graph (dashed surface in Figure SM1) represents the total number of β-cells in the pancreas. This surface was calculated by integrating the curve using Excel software. The result, corrected for the thickness of the sections, gives an estimation of the total number of β-cells per pancreas.

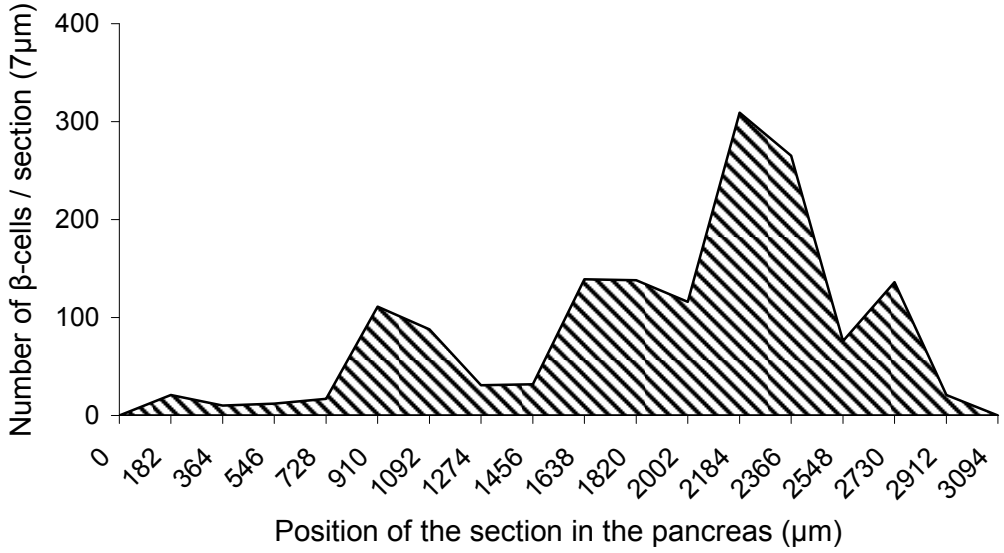


Figure SM1

Supplemental methods II:

Mathematical analysis of β-cell development in control and *hnf6*-/- mice

1) To determine the kinetics of β-cell generation, we started with the data from Figure 1*A* that show the total number of β-cells in control and *hnf6*-/- pancreas at four developmental stages. We added a fifth data point at e14 which corresponds to the onset of β-cell development in wild-type mice. The extended dataset is shown in Table SM1. All developmental stages are represented as days p.c. in order to obtain a continuous scale on the x-axis:

Table SM1

Genotype	Developmental stage (days p.c.)	β-cells / pancreas (mean)	standard deviation	SEM
Control	14	1		
	15.5	11002	2086	1204
	17.5	37986	2744	1584
	23.5	120105	23907	13803
	40.5	340325	68032	39278
hnf6 -/-	15	1		
	15.5	351	192	111
	17.5	3909	966	558
	23.5	12828	1303	752
	40.5	67494	17368	10027

2) Using the "Trend Line Tool" of the Excel software, we plotted the data of Table SM1 in a chart and defined the best fitting trend line for each dataset (Figure 1*D*).

3) We chose the simplest polynomial equations that allowed the trend lines to fit within the standard errors of mean:

$$y_{Ctl} = 7.9842x^2 + 12582x - 182123$$
$$y_{hnf\,6-/-} = 3.4422x^3 - 206.04x^2 + 5578.7x - 49149$$

where *y* is the total number of β-cells per pancreas and *x* the time (days p.c.).

These equations allowed us to predict the total number of β-cells at any given developmental stage.

4) We then calculated the absolute speed of β-cell generation *V* [β-cells/day] which is given by the first time derivative of the total β-cell number *y*:

$$V_{Ctl} = \frac{dy_{Ctl}}{dx} = 15.968x + 12582$$
$$V_{hnf\,6-/-} = \frac{dy_{hnf\,6-/-}}{dx} = 10.3266x^2 - 412.08x + 5578.7$$

5) To compare the kinetics of β-cell formation in control and *hnf6*-/- mice, we used the relative speed of β-cell generation *v* [new β-cells/(day x pre-existing β-cell)] which is given by *V/y*:

$$v_{Ctl} = \frac{V_{Ctl}}{y_{Ctl}} = \frac{(15.968x + 12582)}{(7.9842x^2 + 12582x - 182123)}$$
$$v_{hnf\,6-/-} = \frac{V_{hnf\,6-/-}}{y_{hnf\,6-/-}} = \frac{(10.3266x^2 - 412.08x + 5578.7)}{(3.4422x^3 - 206.04x^2 + 5578.7x - 49149)}$$

6) We then calculated the difference v_Δ between the relative speed of β-cell generation in *hnf6*-/- and control mice:

$$v_\Delta = v_{hnf\,6-/-} - v_{Ctl}$$

This equation was solved numerically (using the Excel spreadsheet-calculation tool) and the v_Δ value was calculated for each day p.c. (Figure SM2). As expected v_Δ is positive and

decreases rapidly during embryonic development: The fact that β-cell generation starts later in *hnf6*-/- mice, translates into a higher *relative* speed when the first β-cells appear. With every newly generated β–cell, this value decreases. If β-cell generation would be identical in *hnf6*-/- and control mice, v_Δ would tend towards 0 with time. However, between 20 and 25 days p.c., v_Δ reaches a minimum and then increases with time, indicating an increase in relative speed of *hnf6*-/- mice as compared to control mice.

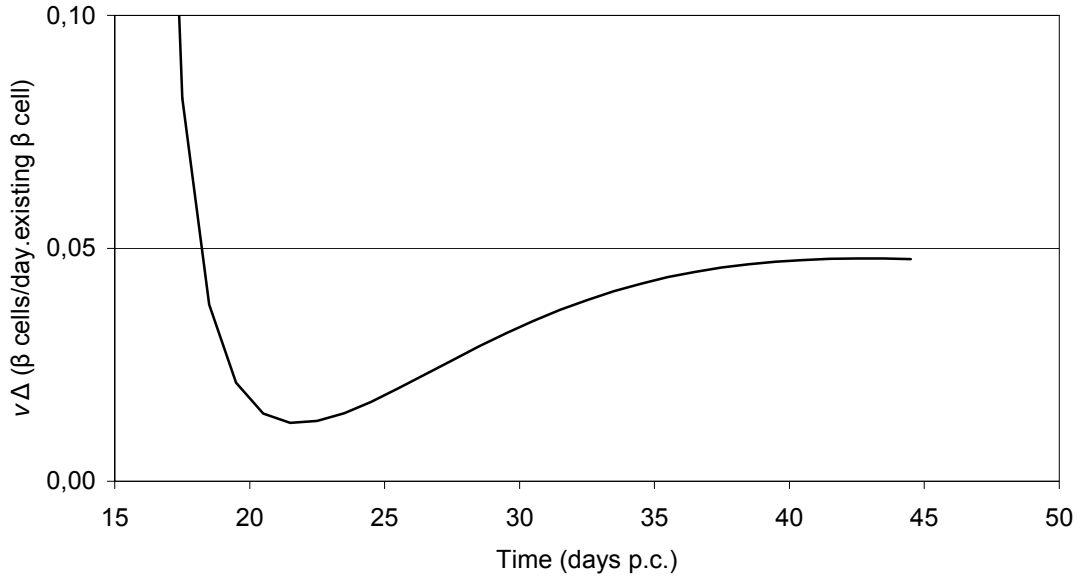


Figure SM2

In order to calculate the contribution of v_Δ to the total production of β-cells / day in the *hnf6*-/- pancreas, we multiplied the relative speed v_Δ by $y_{hnf6\text{-}/\text{-}}$ and obtained V_Δ [β-cells/day], which represents the absolute speed of β-cell formation due to v_Δ.

$$V_\Delta = v_\Delta \times y_{hnf\,6-/-} = v_\Delta \times (3.4422\text{x}3 - 206.04\text{x}2 + 5578.7\text{x} \;\; 49149)$$

By spreadsheet-calculation, we calculated V_Δ value for each day p.c. (Figure 1*E*, dotted black curve).

8) In order to estimate the contribution of endocrine precursors (NGN3+ cells) to V_Δ, we considered that:

i) In control mice, β-cells are generated from NGN3+ endocrine precursors. We therefore considered that the number of β-cells originating from NGN3+ endocrine precursors correspond to y_{Ctl} value calculated before birth (the total number of β-cells per pancreas; see above).
ii) In *hnf6*-/- mice, the formation of β-cells is delayed by 1 day, hence day x in *hnf6*-/- mice corresponds to day x-1 in control mice.
iii) The mean production of NGN3+ endocrine precursors in *hnf6*-/- mice is 10.84 times lower than in control mice (Figure 1*B*, between E15.5 and E17.5).

The mathematical translation of these three assumptions results in the following equations:

$$p_{Ctl} = y_{Ctl} = 7.9842x^2 + 12582x - 182123$$
$$p_{hnf6-/-} = \frac{7.9842(x-1)^2 + 12582(x-1) - 182123}{10.84}$$

where p_{Ctl} and $p_{hnf6-/-}$ represent the estimated total number of β-cells generated by endocrine precursors in control and *hnf6*-/- mice respectively.

9) To calculate the contribution of NGN3+ endocrine precursors to the absolute speed of β-cell formation V_Δ (Figure 1*E*, dotted black curve), we proceeded as described in steps 4) to 7). The result is plotted as a green curve in Figure 1*E*. When this green curve is subtracted from the dotted black curve, we obtain the red curve that represents the number of β-cells/day that are generated in *hnf6*-/- mice independently from NGN3+ endocrine precursors. The red curve reveals that beyond birth β-cells in *hnf6*-/- mice are generated independently from NGN3+ endocrine precursors. This precursor-independent generation of β-cells can be quantified by calculating the proportional contribution of the new β-cells to the total number of β-cells found in *hnf6*-/- (Figure 1*F*). The results indicate that it contributes to 1/3 of all β-cells found in *hnf6*-/- pancreas at 3 weeks.

Comment: For sake of simplicity, we did not take the proliferation rate of β-cells into account. Since this rate is equal or lower in *hnf6*-/- mice than in controls (Figure 1*C*), not including this parameter in the model leads to some underestimation of the neogenic fraction of β-cells in *hnf6*-/- mice.

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3.3. Lineage tracing of pancreatic endocrine precursors in mice deficient in the transcription factor HNF6

3.3.1. Abstract

OBJECTIVE - Mice deficient in Hepatocyte Nuclear Factor 6 (*hnf6-/-* mice) have strongly reduced β-cell numbers at birth. We have shown previously, that after birth, β-cell neogenesis takes place in these mice. Considering that, in normal mice, all endocrine cells derive from endocrine precursors, we wanted to determine by lineage tracing if all the endocrine cells in *hnf6-/-* pancreas, including neogenic β-cells, derive from endocrine precursors.

RESEARCH DESIGN AND METHODS – A *ngn3-cre:rosa26R-eYFP* transgenic mouse line was used to trace NGN3-positive endocrine precursors and their descendants in *hnf6-/-* and control mice. EYFP-labelled β-cells deriving from endocrine precursors were detected by immunostaining and counted.

RESULTS - In a *hnf6+/+* background, 67% of β-cells are EYFP-labelled at birth, and 96.3% after 7 weeks. In *hnf6-/-* mice, these proportions are 79 % and 93.4%, respectively.

CONCLUSIONS – Both in *hnf6-/-* and *hnf6+/+* mice harboring *ngn3-cre and rosa26R-eYFP* reporter constructs, a fraction of β-cells is unlabeled at birth. Seven weeks after birth, the proportion of EYFP-labeled β-cells has increased and tends towards 100% both in *hnf6-/-* and *hnf6+/+* backgrounds. After our

experiments were performed, postnatal mature β-cells were shown to express low levels of NGN3 (and presumably of NGN3-Cre). Therefore it cannot be determined, whether the postnatal increase in EYFP-positive β-cells results from NGN3 (and NGN3-Cre) expression in mature β-cells or from differentiation from NGN3-positive progenitors.

3.3.2. Introduction

In mice deficient in Hepatocyte Nuclear Factor 6 (HNF6), endocrine pancreas development is severely perturbed (Jacquemin et al., 2000). We have shown in the previous section that the relative deficit in endocrine cells at birth decreases postnatally due to β-cell neogenesis resulting from the transdifferentiation of *hnf6*-deficient duct cells.

In normal mice, all endocrine cells were reported to derive from pancreatic progenitors that transiently express high levels of NGN3. This was shown by tracing the fate of the endocrine precursors using *ngn3-cre* mice (Gu et al., 2002; Mellitzer et al., 2004; Schonhoff et al., 2004; Wang et al., 2010). Since neogenic β-cells in *hnf6-/-* mice derive from ducts, we expected that they would not derive from NGN3-expressing precursors. Therefore, we tested this hypothesis by using a *ngn3-cre*:*rosa26R-eYFP* reporter mouse model, in which we expected that β-cells derived from endocrine precursors would express EYFP, but not β-cells derived from duct cells.

3.3.3. Research design and methods

Mice

ngn3-cre reporter and *hnf6-/-* mice (kind gift from P. Herrera) have been described (Herrera et al., 2002; Jacquemin et al., 2000). *ngn3-cre* reporter mice were first crossed with *hnf6+/-* mice. The resulting *ngn3-cre:hnf6+/-* mice were then crossed with *rosa26R-eYFP* (Srinivas et al., 2001):*hnf6+/-* mice to produce *ngn3-*

cre:rosa26R-eYFP:hnf6+/+ control mice and *ngn3-cre:rosa26R-eYFP:hnf6-/-* mice. Mice were sacrificed at 1 day and at 7 weeks after birth to analyze EYFP and insulin expression in the pancreas.

Tissue preparation and immunofluorescence

Tissue samples were fixed overnight at 4°C in 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS). After paraffin embedding, sections were cut to 7 µm. Heat-mediated antigen retrieval was performed in a Tris buffer (10 mM, pH 10) for 4 hours at 60°C in a PT Module (Labvision). All samples were permeabilized in PBS/0.3% Triton X-100 and blocked with PBS/10%BSA/3% milk powder/0.3% Triton X-100. The following primary antibodies were diluted in blocking buffer and incubated overnight at 4°C: guinea pig anti-insulin (DAKO, 1:1000) and rabbit anti-GFP (Abcam, 1:1000). Secondary antibodies (Alexafluor series from Invitrogen) and TO-PRO-3-iodide nuclear stain (Invitrogen, 1:1500) were diluted in PBS/10%BSA/0.3% Triton X-100.

Cell counts

Insulin-positive cells (Ins+) and insulin-EYFP double positive cells (EYFP+/Ins+) were counted on a Zeiss Axiovert 200 fluorescence microscope.

Ethics

All animal experiments were performed according to the institution's ethical guidelines.

3.3.4. Results

We determined the percentage of β-cells derived from endocrine precursors in control and *hnf6-/-* mice, at birth and 7 weeks after birth, using the *ngn3-cre:rosa26R-eYFP* model. After immunolabelling of insulin and EYFP, single and double labeled cells were counted. At 1 day after birth, 66.5% of the Ins+ cells were also EYFP+ in the h*nf6+/+* samples (confidence interval (CI) at P = 0.95: ± 4.5%) (Figure 1A-C,G).

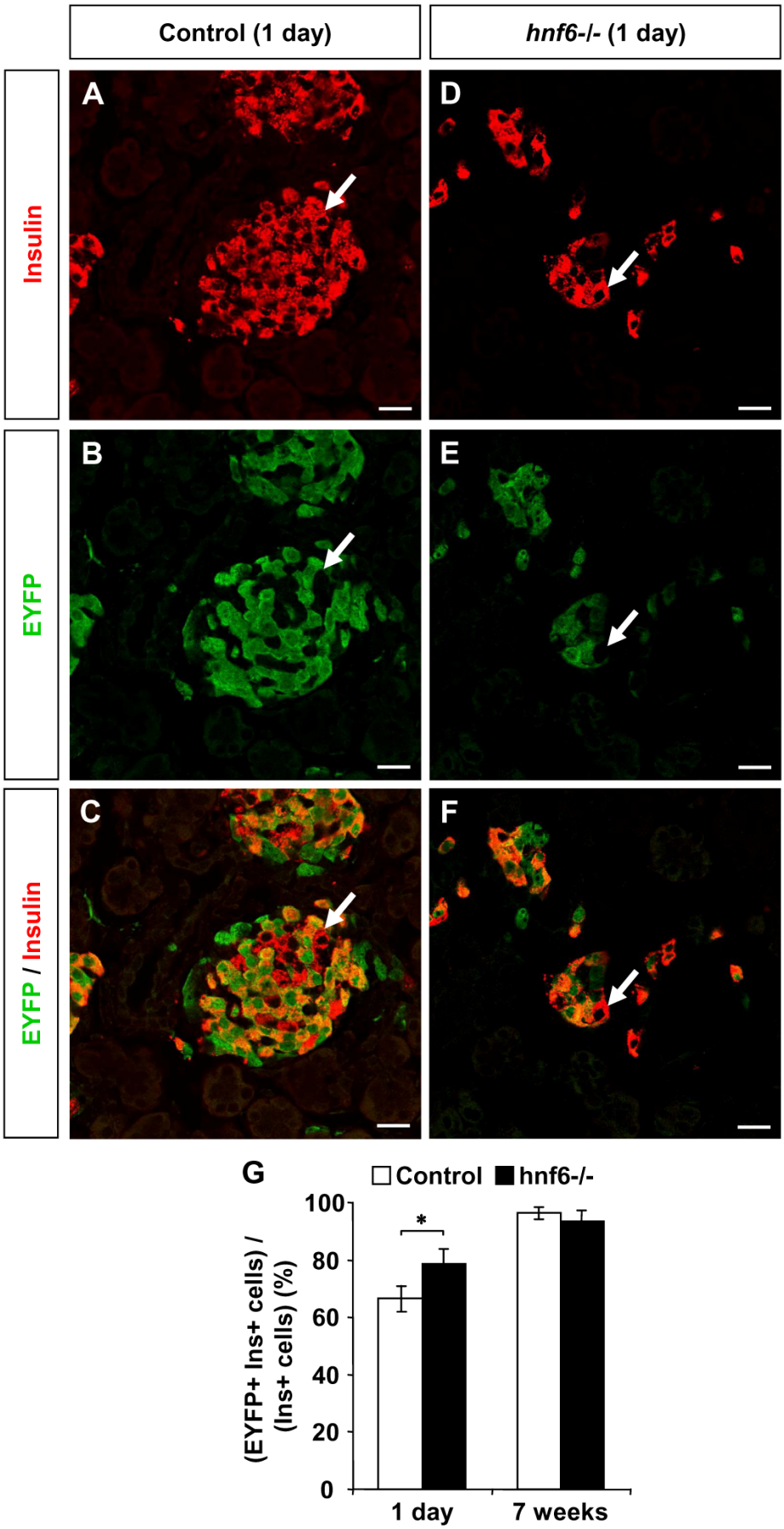
Control (1 day)
hnf6-/- (1 day)
Insulin
EYFP
EYFP / Insulin
A
B
C
D
E
F
G
Control
hnf6-/-
(EYFP+ Ins+ cells) / (Ins+ cells) (%)
100
80
60
40
20
0
*
1 day
7 weeks

Figure 1*. *EYFP/insulin immunolabeling of ngn3-cre:rosa26R-eYFP:hnf6+/+ control and ngn3-cre:rosa26R-eYFP:hnf6-/- pancreas. *(A-F) Labeling at 1 day after birth. EYFP-negative β-cells are indicated by white arrows. Scale bars, 20µm. (G) Percentage of EYFP–positive β-cells at 1 day and seven weeks after birth (*, P = 0.95). The labeling efficiency increases with time and tends towards 100% at 7 weeks. Error bars represent confidence intervals (P = 0.95).*

At the same age, the ratio was higher in the *hnf6-/-* pancreas: 78.6% (CI at P = 0.95: ± 5.2%) (Figure 1D-G). According to a hypothesis test (P = 0.95), this difference is significant.

Seven weeks after birth, the EYFP labeling efficiency in β-cells had increased in control and *hnf6-/-* pancreata and tended towards 100% (Figure 1G). In *hnf6+/+* control samples, 96.3% Ins+ cells were labeled for EYFP (CI at P = 0.95: ± 1.9%). At the same age, the ratio was 93.4% in *hnf6-/-* pancreas (CI at P = 0.95: ± 4.0%). According to a hypothesis test (P = 0.95), the difference between control and *hnf6-/-* samples is not significant at this stage.

3.3.5. Discussion and conclusion

It is commonly accepted that all endocrine cells derive from NGN3-expressing endocrine precursors. This observation was obtained by lineage tracing using *ngn3-cre* mice (Gu et al., 2002). In this publication, at birth and seven weeks after birth, all the islet cells were labeled by the tracer molecule. This was not the case in our study. The discrepancy may originate from the fact that different *ngn3-cre* lines were used. In the study described above (Gu et al., 2002), a 6.5 kb *ngn3* promoter was used to express Cre. In the *ngn3-cre* model used in our experiments, Cre expression was driven by a 4.5 kb promoter fragment (P. Herrera, personal communication).

When our experiments were set up, it was commonly accepted that NGN3 expression was turned off in postnatal mice (Jensen et al., 2000; Schwitzgebel et al., 2000). However, recent findings suggest that low-level expression of NGN3 persists in adult β-cells. This level is sufficient to label β-cells postnatally, using a *ngn3-creER* mouse model (Wang et al., 2009), and is probably also responsible for the

increase in EYFP-positive β-cells detected after birth in our experimental setting. Unfortunately, this does not allow to distinguish if EYFP expression in insulin-positive cells results from postnatal expression of NGN3 in the mature β-cells or from NGN3 expression at an earlier progenitor stage.

3.3.6. References

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4. General discussion and perspectives

In the field of cell therapy of diabetes, the potential use of pancreatic duct cells as a source of new β-cells has been debated for a long time. In vitro studies have suggested a positive answer to this question (Bonner-Weir et al., 2000; Gao et al., 2003; Githens, 1988; Ramiya et al., 2000; Sharma et al., 1999), and a recent lineage tracing of duct cells may reinforce this suggestion (Inada et al., 2008). However, this recent result is weakened by the lack of specificity of the tracer used (Puri and Hebrok, 2010). Moreover, our analysis points out additional weaknesses in this work, namely the surprisingly high proportion of β-cells that would derive from duct cells (see point 1.5 of the Introduction). Finally, recent lineage tracing experiments performed with *hnf1β-creER* and *mucin-creER* tracer mice indicated that duct cells do not give rise to β-cells (Kopinke and Murtaugh, 2010; Solar et al., 2009).

Our present work supports that duct-to-β-cell transdifferentiation occurs in the absence of the transcription factor HNF6, whose postnatal expression is restricted to the duct cells. The same expression pattern has been described for two other transcription factors, SOX9 and HNF1β. In some *hnf6-/-* duct cells, expression of SOX9 and HNF1β is lost, and simultaneous loss of the three factors might contribute to the transdifferentiation process. Together with the misexpression of Pdx1, observed in some *hnf6-/-* ducts (Jacquemin et al., 2000), this could result in cells which have the capability to evolve towards an endocrine fate to a some extent. Thus we speculate that HNF6 plays a role in determination and maintenance of the duct cell fate. In line with this idea, recent unpublished experiments from our laboratory show that forced

expression of HNF6 in cultured acinar cells activates expression of duct markers (Simion et al., unpublished data).

At this stage, it remains unclear whether duct-to-β-cell transdifferentiation in *hnf6-/-* mice is cell-autonomous or not. *hnf6-/-* mice are diabetic (Jacquemin et al., 2000), and conditions such as hyperglycemia could trigger transdifferentiation. However, all *hnf6-/-* pancreata show postnatal β-cell neogenesis although 20-25% of *hnf6-/-* mice do not develop diabetes (Lannoy et al., 2002). To distinguish between the influence of glucose metabolism and a cell autonomous role of HNF6, it would be interesting to investigate if and how β-cell neogenesis occurs in mice with a duct-specific deletion of HNF6. With such a mouse line, the question could be settled by experiments of selective β-cell destruction (e.g. with alloxan or streptozotocin), followed by a regeneration period with or without insulin treatment.

Transdifferentiation could also be triggered by a mesenchymal factor. In *hnf6-/-* pancreata, stromal tissue is present in greater proportions around the ducts. The stromal cells associated with the *hnf6-/-* ducts could be the source of such a factor. Another possibility is that, in *hnf6-/-* mice, a secreted factor released by another tissue could initiate transdifferentiation. HNF6 is also expressed in liver, and it is known that hepatic FGF7/KGF is able to induce the expression of insulin in pancreatic ducts (Nguyen et al., 1996). Production of FGF7 in the liver of *hnf6-/-* mice should be measured to address the potential role of a HNF6-FGF7-pancreatic duct cascade.

The cell autonomous role of HNF6 can also be addressed in in vitro experiments, in which *hnf6-/-* pancreatic duct cells are cultured in conditions that would allow β-cell neogenesis. An option would be to inhibit HNF6 expression in cultured wild-type duct cells by means of HNF6 siRNA.

β-cell neogenesis detected in *hnf6-/-* mice could also originate from pancreatic progenitors that persist in the pancreas and maintain their multipotency after birth. One would then expect that postnatal β-cell neogenesis in *hnf6-/-* mice would proceed according to a differentiation procedure that recapitulates normal β-cell differentiation in the embryo. This would imply the presence of endocrine precursors expressing high levels of NGN3. However, immunohistochemistry did not reveal such postnatal NGN3 expression, and NGN3 mRNA levels were not higher in *hnf6-/-* pancreata than in control organs (see chapter 2.3.). With regard to our NGN3-Cre lineage tracing experiment, one could argue that NGN3 might well be expressed postnatally, at levels

that are too low for immunohistochemical detection, but sufficient to drive endocrine development. However, a recent study demonstrated that achieving high levels of NGN3 expression is a critical step for endocrine commitment from multipotent pancreatic progenitors and that in mice heterozygous or hypomorphic for NGN3, a significant number of the detected NGN3-positive cells adopt ductal or acinar fates (Wang et al., 2010). Hence, NGN3 levels, that are too low to be detected by immunofluorescence, are probably also too low to drive endocrine development. A further argument against NGN3-dependent β-cell neogenesis in *hnf6-/-* mice is provided by results from pancreatic duct ligation experiments. While duct ligation in wild-type mice leads to activation of NGN3-positive precursors (Xu et al., 2008), no NGN3 expression was detected in *hnf6-/-* mice in the same conditions (Xu et al., unpublished data). Such observations make it rather unlikely that postnatal β-cell neogenesis in *hnf6-/-* mice results from delayed differentiation of pancreatic progenitors.

We also tried to answer the question of the NGN3 role by generating *hnf6-/-:ngn3-/-* double knockout animals. Unfortunately, these animals die at birth, before the production of the first neogenic β-cells (see section 3.2.4). Obtaining a definitive answer to the question whether β-cell neogenesis in *hnf6-/-* pancreas is NGN3-dependent will require the generation of *hnf6-/-* mice with duct cell-specific ablation of NGN3. It then could be tested if β-cell neogenesis still occurs. However, there might be limitations to such experiments if NGN3 is not eliminated in all duct cells.

It has recently been shown that α-cells transdifferentiate spontaneously to β-cells after near-total ablation of the latter (Thorel et al., 2010), suggesting that such a mechanism could contribute to β-cell neogenesis in *hnf6-/-* mice. During α- to β-cell transdifferentiation, transient cells co-expressing glucagon and insulin are observed (Thorel et al., 2010). In *hnf6-/-* mice, such cells are not observed (Jacquemin et al., 2000), making it unlikely that the mechanism operating in the conditions described by Thorel et al. is functional during the β-cell neogenesis in the *hnf6-/-* pancreas. In addition, the balance between α- and β-cells does not fluctuate in an important manner over time in *hnf6-/-* mice. This suggests that glucagon cells could also be produced from duct cells in these mice,

The observation that only a fraction of *hnf6-/-* duct cells give rise to β-cells suggests that the lack of HNF6, SOX9, and HNF1β is not sufficient to induce massive

transdifferentiation. The loss of additional transcription factors or signaling pathways could further promote duct cell transdifferentiation. One candidate is the Notch signaling pathway. Indeed, Notch mutants do not have any cells positive for duct markers (Lorent et al., 2004; Yee et al., 2005), and when Notch signaling is activated in acini, it initiates and promotes the formation of ductal intraepithelial neoplasia (De La O et al., 2008). In addition, during development, Notch signaling not only prevents endocrine development (see section 1.3.2.) but also appears to play a positive role in duct formation (Greenwood et al., 2007). PAX4 is a marker of committed endocrine cells downstream of NGN3, and when Notch activity is forced in PAX4-positive cells, more pancreatic ducts are formed. Therefore, active Notch signaling is associated with promotion of duct differentiation. Moreover, it has been shown that pancreatic duct hypertension activates the Notch signaling pathway in dilated ducts (Bhanot et al., 2008). A similar situation might be found in the cysts and dilated ducts of *hnf6-/-* mice. We speculate that, in *hnf6-/-* ducts, Notch signalling might be active and represent a brake to a more massive duct cell transdifferentiation. Such a hypothesis is further supported by the observation that inhibition of the Notch pathway in duct-like cultures increases the production of β-cells (Baeyens et al., 2009). Hence, inhibiting the Notch pathway in *hnf6-/-* duct cells is expected to further promote β-cell differentiation.

Considering our results, it should be interesting to test if in vitro production of β-cells could be achieved by inhibiting HNF6, and possibly HNF1β and SOX9, expression in primary cultures of duct cells. Such inhibition could be coupled with other treatments known to have a positive influence on the formation of insulin-producing cells. The functionality of the β-cells could then be tested by grafting in diabetic mice.

In summary, our study shows that a postnatal compensatory growth of β-cells occurs in *hnf6-/-* mice, which suffer from severe β-cell deficiency at birth. This compensatory growth results neither from increased proliferation, nor from differentiation of NGN3-positive precursors. Lineage tracing experiments in *hnf6-/-* mice provide evidence for duct-to-β-cell transdifferentiation. The understanding of the underlying mechanism could help developing interesting tools for in vitro generation of therapeutic β-cells from expanded ductal tissue or for the in vivo replenishment of a deficient β-cell pool in diabetic patients.

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A promising way to cure diabetes is to implant in vitro produced β-cells, or to induce β-cell regeneration in vivo. Multiple attempts have been made to coax different cell types into β-cells. One of these cell types is the pancreatic duct cell, but its potential as a β-cell precursor remains unclear.

In this work we examine the plasticity of duct cells in mice deficient for Hepatocyte Nuclear Factor 6 (HNF6), a transcriptional regulator of pancreatic cell differentiation. In *hnf6*-/- mice, the number of β-cells is reduced at birth, yet it subsequently undergoes a partial recovery. We show in these mice that a postnatal β-cell neogenesis occurs. Neogenesis neither results from a higher proliferation rate of mature β-cells nor from delayed differentiation of β-cell precursors. The presence of transition cells that co-express insulin and duct markers, as well as genetic lineage tracing, indicate that duct cells transdifferentiate postnatally to β-cells in *hnf6-/-* mice.

Therefore, our results provide evidence that in the absence of the transcription factor HNF6, duct cells have the potential to generate β-cells. This suggests new strategies for the production of β-cells from duct cells.